

LAND MANAGEMENT AFFECTS MICROBIAL COMMUNITY COMPOSITION  
AND FUNCTION IN CARBON CYCLING

A Dissertation

Presented to the Faculty of the Graduate School  
of Cornell University

In Partial Fulfillment of the Requirements for the Degree of  
Doctor of Philosophy

by

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August 2016

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# LAND MANAGEMENT AFFECTS MICROBIAL COMMUNITY COMPOSITION AND FUNCTION IN CARBON CYCLING

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Cornell University 2016

Microbes are essential for soil carbon cycling, mediating an estimated 90% of decomposition in soils. Evidence exists for the influence of microbial community composition on community function in soil carbon cycling. However, we are still learning about the impact of season and land management on community structure. Here, I use high-throughput sequencing to characterize changes in microbial community composition across tillage, biomass management, and time. Additionally, I use high resolution stable isotope probing (HR-SIP) to test the relationship between community structure and function in cellulose and xylose decomposition across tillage managements. I find that tillage, biomass management, time, and interactions between these variables affect both bacterial and fungal communities. Soils experiencing no-till and returned biomass management contain OTUs that increase significantly in abundance during the fall and early spring, while soils experiencing combinations of tillage and biomass management or no-till and biomass removal do not. I hypothesize that the return of biomass, combined with the soil structure and baseline carbon content from no-till, biomass return conditions creates an environment supportive of the growth of these responsive OTUs. Additionally, I find evidence for differences in OTUs incorporating carbon from cellulose and xylose across tillage managements early in the decomposition process for xylose and cellulose (days 1-3 for xylose and days 3-7 for cellulose). These differences correspond with differences in CO<sub>2</sub> production between till

and no-till soils, with higher CO<sub>2</sub> production in no-till soils. Together, these results offer support for the functional significance of microbial communities in carbon cycling. Further understanding the impact of land management, time, and interactions between these variables on community structure aids in greater understanding of microbial communities in the soil and their function in soil carbon cycling. This information may provide support for inclusion of microbial community structure parameters into models of soil carbon fluxes.

## BIOGRAPHICAL SKETCH

Chantal Nathalie Koechli attended college at the College of Wooster in Wooster, Ohio, where she majored in Biochemistry and Molecular Biology with a minor in French. At Wooster, she was advised by Dr. Stephanie Strand, Dr. Melissa Schultz, and Dr. Mark Snider and did independent research characterizing degradation of antidepressants in wastewater sludge. After college, Chantal spent a year in France teaching English at a high school through the Teaching Assistant Program in France (TAPIF), before beginning graduate school in Microbiology at Cornell. She joined Dr. Daniel Buckley's lab in the fall of 2011. This fall, she will join the faculty at Beloit College as a Visiting Assistant Professor of Biology.

Dedicated to my parents, Heidi and Toni Koechli, who have always supported me.

## ACKNOWLEDGMENTS

I'd like to thank my parents and sister, Vanessa, for always supporting and loving me in my educational and life journey. Thanks to Daniel Buckley, who embodies the curious scientist, has been very supportive of independent exploration of new ideas, and is always up for questions and scientific conversation. Many thanks also to my labmates, Nick, Chuck, Spencer, Mallory, Ashley, Anay, Sam, and Kevin, for stimulating conversation (scientific and non) and support and encouragement for the days when science was hard. You made Bradfield much homier. I am indebted to the helpful staff at the Miner Institute, especially Mike Davis, who always ok'd my trips and gave me useful weather advice. I am also very grateful for friends at Cornell and in Ithaca, including Olga, Marc, Vaidehi, Naman, Amy, Jake, Aravind, Dave, Eugenia, Jeff, Angela, Flossie, who always lent an ear, a hand, and a smile with many good times shared. Finally, I'd like to thank my husband, David, for his constant encouragement, support, and love. He has been a ever present help these five years: sampling soils in Chazy during snowstorms, dealing with lab-stress, listening to countless talks and ideas, and getting married 10 days before a thesis defense. I couldn't have done it without you.

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# **Introduction**

## **1.1 Background**

Humans have explored the mysteries of space, yet we often overlook a system that is similarly enigmatic, existing just underneath our feet. Even in this time of scientific and technological advancement, the words of Leonardo daVinci from the 16<sup>th</sup> century still ring true, “We know more about the movement of celestial bodies than about the soil underfoot.” This is partially due to the enormous complexity of the soil environment, including the diverse microbial community that exists and carries out life-sustaining nutrient cycling in the soil. It is estimated that there may be as many as one billion bacterial cells per gram of soil, estimated to account for 1,000-100,000 different genomes. However, less than 1% of these have been cultured (Gans et al., 2005; Nannipieri et al., 2003; Roesch et al., 2007; Torsvik et al., 1990; Tringe et al., 2005). Given that these organisms have been evolving for billions of years, their various functions and physiologies are incredibly diverse. Our dearth of knowledge is wide, including the intricacies of their role in soil carbon cycling.

## **1.2 Soil Carbon Cycling and the Soil Microbial Community**

The carbon cycle is one of the essential biogeochemical cycles that sustain life on earth. Soil globally stores 2500 Pg of carbon as soil organic matter, receives around 60 Pg C / year of plant biomass, and releases about 60 Pg C/ year of carbon to the atmosphere through respiration (IPCC, 2014). Within the soil, bacteria and fungi are responsible for about 90% of all organic matter decomposition (McGuire and Treseder,

2010). However, these microbes do not function in isolation, but instead are members of larger ecosystems that play important functional roles in soil carbon cycling (Brussaard, 1997; DeAngelis et al., 2008).

Soil is poor in nutrients and energy sources, and these components are distributed non-uniformly, producing micro-scale levels of heterogeneity (Nannipieri et al., 2003; Schimel et al., 2005; Schmidt et al., 2011). Therefore, microbial habitats also exist non-uniformly in discontinuous planes. The existence of microbial habitats depends not only on carbon and energy sources, but also on nutrient availability, ionic composition, water availability, temperature, pressure, air composition, electromagnetic radiation, pH, oxidation-reduction potential, and structure (aggregates, pores) of the soil environment (Foster, 1988; Nannipieri et al., 2003; Ranjard and Richaume, 2001). The discontinuous microbial habitats that form can sometimes become continuous, linked by water bridges after precipitation events, increasing interactions and complexity (Nannipieri et al., 2003; Ranjard and Richaume, 2001). With all of the connections between the microbial community and other abiotic and biotic factors, unraveling soil carbon cycling dynamics becomes a complicated task.

### **1.3 Anthropogenic Disruption of Carbon Cycling**

Humans also play a role in the soil carbon cycle, albeit an unbalanced one. Anthropogenic emissions add around 9 Pg of carbon into this cycle each year, mainly through combustion of fossil fuels (Canadell et al., 2007). Around 3 Pg of this carbon is stored in terrestrial ecosystems, around 2 Pg is absorbed by oceans, while 4 Pg is left in the atmosphere (Canadell et al., 2007). The increased C in the atmosphere, in the form

of CO<sub>2</sub> and CH<sub>4</sub>, contributes to greenhouse warming and climate changes associated therewith. In addition to fossil fuel combustion, humans produce much of their food through agricultural means. Agriculture disrupts natural soil cycling, through removal of plant biomass and nutrients and land management such as tillage (McLauchlan, 2006).

Through the agitation and turnover of soil, tillage eases weed pressure and facilitates planting, promoting short-term gains in agricultural productivity (Govaerts et al., 2009; Lal, 2009). Over time, though, tillage can contribute to a loss of organic matter, reduced soil aggregation, erosion, and compaction in soils (Govaerts et al., 2009; Lal, 2009). An alternate land management practice is no-till agriculture, which refers to the agriculture practice in which soils are not disturbed (Govaerts et al., 2009). A meta-analysis of conventional tillage compared to zero till (no-till) management have shown that in the majority of cases, the conversion of fields from conventional tillage to no-till results in an increase in soil organic carbon (SOC) stocks (Govaerts et al., 2009). However, the variable of tillage does not alone determine SOC stock outcome. Other factors, such as climate and physical properties of soils (bulk density, quantity of small macropores), can impact the effect of till or no-till on soil carbon pools (Govaerts et al., 2009). Even the depth sampled can impact the evidence for tillage effect on soil carbon pools (Dolan et al., 2006). In some cases, conversion to no-till does not show evidence for increase soil carbon pools (Dolan et al., 2006; West and Post, 2002). Therefore, although much evidence supports the increase in SOC in no-till management, there are exceptions. No-till management also can have higher soil respiration than till managements, but these fluxes can also be dynamic (Attard et al., 2016; Mbuthia et al.,

2015; Oorts et al., 2007). Soil C emissions might fluctuate in no-till vs. tilled soils at different points of the year, due to effects of plant C inputs, temperature, moisture, and other seasonal factors that interact with tillage (Oorts et al., 2007).

There is strong evidence for increased soil aggregation, moisture, and reduced erosion under no-till management, as compared to till management (Govaerts et al., 2009). Each of these characteristics is thought to increase overall soil health (Lal, 2009). Additionally, no-till management is considered more cost-effective than conventional tillage, due to the reduction of inputs (fertilizers, fossil fuels costs) and evidence of long-term higher yields (Govaerts et al., 2009; Lal, 2009). Thus, current opinion is that no-till management is better for soil health, agricultural sustainability, and long-term crop yield as compared to conventional till practices (Govaerts et al., 2009; Lal, 2009).

Research support, though, does not always translate to adoption. As of 2010-2011, around 40% of combined corn, soybean, wheat, and cotton acreage in the United States was managed under conservation tillage practices (either no-till or strip-till – only tilling the strip used for planting) (Wade, Tara et al., 2015). For corn cropping, just over 30% of total acreage was under conservation tillage (Wade, Tara et al., 2015). Thus, the majority of land in the U.S. remains farmed under till conditions.

In addition to tillage, biomass management can impact soil carbon pools and soil carbon cycling. Biomass management refers to the retention or removal of plant biomass (ex. corn stover) on agricultural fields. Plant biomass removed from fields can be used for biofuel production, for a potential monetary benefit (Lal, 2009). However, removal of the plant biomass prevents the return of carbon, nitrogen, and other nutrients

to the soil (Hill et al., 2006; Lal, 2009). Over time, continuous removal of biomass can produce soils that are considerably depleted in C and N, leading to poorer yield returns (Cherubini et al., 2009). Retention of plant biomass counters soil nutrient depletion. As research and investment in cellulosic biofuels has increased sharply over the past years, so has the amount of cropland that grows plants for biofuels purposes, and thus is managed under biomass removal (Lal, 2009).

In an ecological sense, the disruption of soil carbon cycling due to agricultural practices, such as tillage and biomass removal, may have significant repercussions on the stability and usage of these ecosystems. These ramifications may be especially detrimental for humankind, when considering consequences linked to reduced soil health, including reduced agricultural yield, and increase in carbon loss in response to global change (Marennya and Barrett, 2009; Schmidt et al., 2011).

#### **1.4 Modeling Soil Carbon Cycling – do Microbes Matter?**

It is unclear how terrestrial carbon storage will change with increasing temperature and altered climate. Predictions from global climate models vary widely (Friedlingstein et al., 2006; Wieder et al., 2013). These models are unable to represent variance in present and past data, suggesting that they are lacking essential terms (or even categories of terms). Recent evidence implicates global C-models as underestimating the role of microbial community composition and function in soil carbon cycling (McGuire and Treseder, 2010; Treseder et al., 2012). Studies that include microbial-related parameters (ex. biomass and carbon use efficiency) into global carbon models have shown an increase in variance explained (Graham et al., 2016; McGuire

and Treseder, 2010; Trivedi et al., 2013; Wieder et al., 2013). Further study of the role of microbial communities in carbon cycling may determine and validate microbial parameters of importance for carbon models, as well as helping with predictions of future impacts on soil cycling.

Tillage and biomass management impact structure of microbial communities (Caesar-TonThat et al., 2010; Drijber et al., 2000; Jansa et al., 2003; Mbuthia et al., 2015; Navarro-Noya et al., 2013; Spedding et al., 2004). However, it is not yet clear whether microbial community response to land management may be linked to changes in soil carbon cycling, that is, whether the structure of microbial communities may impact their function in soil carbon cycling. Therefore, it is unclear whether inclusion of parameters such as microbial community structure may be important to consider when explaining or understanding carbon cycling dynamics under different agricultural land managements. Additionally, little is known about the seasonal changes in microbial community in different land managements and how interactions between time and land management might affect community structure. The work presented in this thesis seeks to address these unknowns.

## **1.5 Sequencing-Based Approaches as Tools to Characterize Soil Microbial Communities and Carbon Cycling Under Differing Land Management Strategies**

In the past decade, incredible advances have been made in culture-independent, high throughput methods for characterizing microbial communities. One of the most impactful of these tools is high-throughput sequencing (Caporaso et al., 2012), which allows for the identification of individuals within microbial communities through the

sequencing of markers such as small subunit (SSU) RNA genes. This method allows for the processing of hundreds of samples at a time and produces millions of sequences (Caporaso et al., 2012; Kozich et al., 2013). The deep level of sequencing facilitated by high-throughput sequencing allows identification of individual OTUs that differ across land managements or other variables of interest. However, high-throughput sequencing of a marker gene, such as the SSU RNA gene, only provides information about the identity of an organism but does not provide insight into function. The technique of stable isotope probing, combined with high throughput sequencing, is a powerful tool that links microbial identity with function.

In stable isotope probing, an isotopically-labeled substrate is added to a system under certain experimental conditions, time is allowed for incorporation of the isotope into biomass, and then isotopic centrifugation and fractionation are undertaken to separate labeled biomass from unlabeled biomass (Lueders et al., 2004a; Radajewski et al., 2000). This technique allows for researchers to track a substrate central to carbon cycling, such as cellulose, throughout the soil microbial community under a variety of conditions. SIP has been successfully used to track contaminants, such as naphthalene and phenol, through microbial communities, as well as substrates central to carbon cycling, including glucose, cellulose, and methanol (DeRito et al., 2005; Eichorst and Kuske, 2012; Lueders et al., 2004b; Padmanabhan et al., 2003). High-throughput next generation sequencing then allows for the rapid and cost-effective identification of microbes that take up the substrate of interest, enabling us to begin to map the network of carbon cycling in the soil (Bartram et al., 2011; Caporaso et al., 2012).

## **1.6 Summary and Introduction of Work**

High-throughput sequencing is a tool that allows for in-depth probing and characterization of microbial communities in response to land management, sampling date, and their interactions. Sequencing combined with stable isotope probing allows us to begin testing the functional significance of the microbial community involved in carbon cycling across soils of different land managements. In chapter two, I characterize the bacterial community in response to land management and time and identify important interactions, resulting in seasonal changes in community structure. In chapter three, the same is done for the fungal community. Finally, in chapter four, I characterize the impact community structure on function of the bacterial community involved in cellulose and xylose metabolism across different tillage managements.



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## 2 Chapter 2 : Changes in Bacterial Communities are Driven by Interactions between Land Management and Season

### 2.1 Abstract

Soil microbial community composition differs across land management and season. Interactions between land management and temporal variation can also contribute to compositional differences in microbial communities. However, studies rarely explore these interactions over long periods of time, even though such knowledge may better predict carbon fluxes from soils. Using high-throughput Illumina MiSeq sequencing, we characterized the bacterial community across tillage, biomass management, and season at a long-term (42 year) field site over the course of two years. Season, tillage, and biomass management impacted bacterial community composition. We also identified significant interactions between biomass management and season on community composition (specifically in the no-till, biomass-retained management). OTUs from *Proteobacteria* (orders: *Caulobacteriales*, *Sphingomonadales*, *Oxalobacteriales*, *Pseudomonadales*, and *Xanthomonadales*), *Bacteroidetes* (families: *Cytophagales*, *Flavobacteriales*, *Chitinophagales*, *Sphingobacteriales*), *Verrucomicrobia* (family: *Verrucomicrobiales*), and *Actinobacteria* (family: *Microbacteriales*), may be driving this interaction by increasing in abundance in response to changing carbon supply to the bacterial community. Increased comprehension of bacterial community dynamics across land management, time, and their interaction provides greater understanding of bacterial niches and their role in soil carbon cycling.

## 2.2 Introduction

Agricultural land management can have a large impact on soil structure, organic matter content, microbial biomass production and plant growth (Filser et al., 1995; Gispert et al., 2013; Grandy et al., 2009; Lee and Schmidt, 2014; Wickings et al., 2011). Tillage and organic matter biomass management are major components of land management practices. In a majority of studies, the use of conventional tillage shows an over 50% reduction of carbon stocks in the soil (Davidson and Ackerman, 1993; Guo and Gifford, 2002), reduces soil aggregation, and promotes erosion (Govaerts et al., 2009). Biomass removal from agricultural fields also reduces carbon stocks and soil aggregation (Turmel et al., 2015). Conversely, in many studies, the practices of no-till agriculture and return of crop biomass to soil may help build carbon pools, increase soil aggregation, and reduce erosion (Govaerts et al., 2009). Thus, no-till agriculture and biomass retention are considered components of sustainable agriculture (Hobbs et al., 2008).

Land management can also affect soil microbial communities. Microbial communities in soil play an essential role in nutrient cycling and organic matter management in soils (McGuire and Treseder, 2010; Nannipieri et al., 2003). Previous studies have found that microbial community composition does differ due to tillage practice and organic matter management (Babujia et al., 2010; Drijber et al., 2000; Frey et al., 1999; Lupwayi et al., 2001; Mbuthia et al., 2015; Navarro-Noya et al., 2013; Ramirez-Villanueva et al., 2015; Spedding et al., 2004).

Soil bacterial communities also exhibit seasonal and temporal variation (Bardgett

et al., 1999; Bossio et al., 1998; Grayston et al., 2001; Matulich et al., 2015). Research conducted over multiple years at a global change experiment (testing CO<sub>2</sub>, warming, nitrogen addition, and water addition) found that interannual variation in microbial communities was of a larger magnitude than experimental treatment effects (Gutknecht et al., 2012). Shorter-term studies, over 2 – 3 years, identify high variability in community composition both within (seasonal) and across years (interannual), but this variability does not mask local environmental differences (Buckley and Schmidt, 2003; Lage et al., 2010). Season-dependent changes of microbial community composition are strong when environmental variables measured also vary seasonally, suggesting interaction of community structure and environmental variables (Matulich et al., 2015).

Because soil organic matter pools change over the scale of years (Attard et al., 2016) and may take decades to impact soil microbial community structure (Buckley and Schmidt, 2001, 2003), long-term studies are necessary to evaluate interactions between land management, soil organic matter dynamics, and microbial communities. However, there have been few long-term studies that have examined compositional differences due to land management, time, and their interaction. The composition of soil microbial communities may contribute to variation in soil C fluxes (Treseder et al., 2012; Wieder et al., 2013). Thus, identifying the specific effects of land management and time on soil microbial communities may also be important for soil carbon modeling and for greater understanding of microbial ecology in soil. Additionally, differential distribution of specific groups of microbes under differing land management conditions over time may provide evidence for ecological niches (Philippot et al., 2010).



Using a long-term (42 year) field site with combined treatments of till or no-till and biomass removal or return, we explored the impacts of land management on composition of bacterial communities over a two-year sampling period. We sequenced the SSU rRNA genes of bacterial communities in each of four treatments (1. Till, removed biomass, 2. Till, returned biomass, 3. No-till, removed biomass, 4. No-till, removed biomass) over eleven time points, which allowed for identification of community-level differences with land management, time, and their interaction. Additionally, sequencing data allowed us to identify specific groups of bacteria that differ due to land management, temporal dynamics, or their interaction.

## **2.3 Methods**

### **2.3.1 Soil Sampling**

We sampled soils from a long-term continuous corn tillage experiment, established in 1973, at the Miner Agricultural Research Institute in Chazy, NY (Clinton County, 44°53.13'N, 73°28.40'W). Experimental plots at the site all contain the same soil type – Raynham silt loam – and are part of a 2 x 2 factorial design that tests both tillage and maize biomass management. Thus, there are four effective treatments: tillage (moldboard plowed and disked) with biomass returned (PTR), tillage with biomass harvested (PTH), no-till with biomass returned (NTR), and no-till with biomass harvested (NTH). Within the biomass returned treatment, grain is harvested but all other plant biomass is returned to the soil. The total plant biomass returned to the soil is around 8.4 – 8.5 Mg ha<sup>-1</sup> (Moebius-Clune et al., 2008). In the harvested treatment, plant biomass is removed for silage by cutting stalks adjacent to the soil surface, with only

roots remaining. Plots (6 x 15.2 m) are arranged in a randomized complete block, with four replicates for each treatment. Maize is fertilized according to standard agronomic practices for the region (26 kg Ha<sup>-1</sup> per year fertilizer N). Harvest occurs in mid-October to early November and tilled plots are tilled by mid-November.

We took soil samples from all replicate plots on over 16 months from July 2014 – November 2015 : 7/10/2014, 9/24/2014, 10/29/2014, 11/26/2014, 4/30/2015, 6/17/2015, 7/16/2015, 8/13/2015, 9/23/2015, 10/27/2015, 11/25/2015. For each plot, we collected 10 cores (2.5 cm diameter, 5 cm depth) along a transect that spanned the complete length of the plot. We also collected soil moisture and temperature data, using moisture and temperature probes, for three points within each plot (beginning, middle, and end of plot), and we averaged across the three points when analyzing data. Samples were transported on ice and cores for individual plots were homogenized and sieved to 2 mm. Once sieved, samples were stored at -80°C.

Additional soil analyses, including pH, soil C (in units of %), and soil N (in units of %) were done for one time point (9/24/2014). We measured pH using standard 1:1 soil-water method, and total soil C and N were determined on oven dried and ground samples via LECO Treu Mac CN-2000\* elemental analyzer (LECO Instruments, Lansing, MI) as previously described (Berthrong et al., 2013)

### **2.3.2 DNA Extraction**

We extracted DNA from two subsamples of 0.25g of soil for each plot at each time point sampled using a modified Griffith's phenol-chloroform DNA extraction method (Griffiths et al., 2000; Pepe-Ranney et al., 2015). Duplicate extracts for each plot-time

point were combined and quantified using a PicoGreen assay (Invitrogen, Carlsbad, CA, USA).

SSU rRNA gene amplicons were generated from DNA by PCR using primer set 515f/806r. Primers were barcoded using the dual indexing scheme of Kozich et al. (Kozich et al., 2013). Each 25  $\mu$ L reaction contained 1x Q5 High Fidelity PCR Master Mix (New England Biolabs), 0.3  $\mu$ M of each primer, 1.25  $\mu$ L BSA, and 0.625  $\mu$ L of PicoGreen reagent (for detection of successful amplification via fluorescence on qPCR machine). Reactions contained a normalized 5 ng of template, and each sample was amplified in triplicate. Thermal cycling occurred with an initial denaturation step of 2 minutes at 95°C, followed by 30 cycles of amplification (20s at 95°C, 15s at 55°C, 10s at 72°C), and a final extension step of 5 min at 72°C. Triplicate amplicons were pooled. Purification and normalization was done using the SequalPrep normalization kit (Invitrogen), using manufacturer's protocol. Samples were concentrated down to 5 ng/ $\mu$ L, via vacuum centrifugation, and were sent to the Cornell Core Facility in Ithaca, NY to be run on an Illumina MiSeq, using the V2 chemistry with 2 x 250 bp read length.

### ***2.3.3 Post-Sequencing Analysis***

#### *Quality Control*

All code used for data processing and analysis can be found on Github ([https://github.com/chvtk/Chazy\\_Bulk\\_515-806](https://github.com/chvtk/Chazy_Bulk_515-806)).

Raw Illumina sequencing reads were merged with PEAR, using program defaults, and then demultiplexed (Zhang et al., 2014). Illumina sequences were then

screened by maximum expected errors, with reads exceeding a maximum expected error threshold of 1.0 being discarded. Alignment-based QC, using Mothur and a representative set from the Silva database (97% identity, version 111), was performed and sequences of lengths less than 253 bp and sequences with homopolymers greater than 8 bp were removed (Kozich et al., 2013).

#### *OTU Clustering and Taxonomic Assignment*

The dataset underwent OTU clustering using UParse (Edgar, 2013). Cluster seeds were identified with a non-redundant set of reads, using USearch version 7.0.1090, with a sequence identity threshold for a new OTU centroid set at 97%. With USearch/UParse, potential chimeras were identified during OTU centroid selection and could not become cluster centroids, eliminating chimeras from the read pool. All quality controlled reads of the combined dataset were then mapped to cluster centroids at an identity threshold of 97%, again using USearch. 67% of quality controlled reads were mapped to centroids. Unmapped reads did not count towards sample counts and were removed from downstream analyses.

Reads were taxonomically annotated using the UClust taxonomic annotation framework in the QIIME software package with cluster seeds from the SILVA SSU rRNA representative set using 97% sequence identity OTUs as reference (release111) (Caporaso et al., 2010; Edgar, 2010; Pruesse et al., 2007). Reads annotated as “Chloroplast”, “Eukarya”, “Archaea”, “Unassigned” or “mitochondria” were removed from the dataset.

### *Rarefaction*

Prior to statistical analyses, samples were rarefied to an even depth of 15,000 sequences per sample. We chose to rarefy as there was a greater than 4-fold difference in sequencing depth among samples (Weiss et al., 2015).

R version 3.1.2 was used for all analyses, utilizing the phyloseq, vegan, dplyr, DEseq2, ggplot2, picante, and MetagenomeSeq libraries (Love et al., 2014; McMurdie and Holmes, 2013; R Core Team, 2014; Wickham and Chang, 2015; Wickham et al., 2015).

### *NMDS Ordination*

Weighted UniFrac used to calculate a phylogenetic distance matrix for samples (Lozupone et al., 2006). The distance matrix was visualized using Nonmetric Multidimensional Scaling (NMDS). Adonis statistics (Permutational Multivariate Analysis of Variance) were used to partition ordination variance attributed to various treatment conditions.

### *Differential Expression Statistical Framework*

The differential expression statistical framework (DEseq2) was used to identify OTUs that were enriched in different treatment conditions (Anders and Huber, 2010; McMurdie and Holmes, 2014). “Differential abundance” refers to different sample means for an individual OTU in contrasting treatments (ex. No-till vs till). DESeq2 was used to calculate the moderated log<sub>2</sub>-fold change of no-till:till and organic matter harvested:organic matter removed, and August:November, August:April, and April:November proportion mean ratios and corresponding standard errors (Anders and

Huber, 2010). P-values were corrected for multiple comparison with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995).

As a form of independent filtering (Bourgon et al., 2010), a sparsity filter was applied to the dataset. A sparsity filter removes OTUs with sequence counts that fail to reach a user defined sparsity threshold. We tested sparsity thresholds from 0.05 – 0.95 in steps of 0.05. A threshold of 0.25 was chosen, as it returned the largest number of significantly differentially enriched OTUs, as per previous protocol (Pepe-Ranney et al., 2016). Thus, any OTUs that were present in 25% or fewer samples were removed from the dataset (Supplemental Figure 2.1). OTUs from the dataset were defined as differentially enriched if they had an adjusted p-value less than the false discovery rate of 0.10 (Pepe-Ranney et al., 2016)

### *Time Series Analyses*

We conducted smoothing-spline ANOVA time series analysis using the `fitTimeSeries` function from the R package `MetagenomeSeq`. We compared abundance over time in individual OTUs for both tillage (till vs. no-till) and biomass (harvested vs returned) treatment. P-values were corrected for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). We considered time-intervals of enrichment to be significant if adjusted p-values were less than 0.01. (Paulson et al., 2013)

### *NTI/NRI Calculation*

Net relatedness index and nearest taxa index, measures of phylogenetic relatedness of a community, were calculated for communities of OTUs enriched under

till, no-till, biomass returned, and biomass removed conditions. For the analysis, we used the R package *picante*, using the null model “taxa labels” (randomly shuffles distance matrix labels) and performing 1000 iterations of 1000 randomizations.

## **2.4 Results**

We sampled a long-term experimental site that varied tillage and biomass retention over the course of two years. We sought to explain variation in bacterial community composition under four land management treatments: no-till, organic matter harvested (NTH); no-till, biomass returned (NTR); plow-till, biomass harvested (PTH); and plow-till, biomass returned (PTR). To do so, we monitored soil bacterial community composition across treatments and time and also measured various soil geochemical traits. This information was used to detect and identify sources of variation in microbial communities, at both community and OTU level, in different treatments and over time.

Time (seasonal and interannual) and tillage were the strongest sources of variation within the bacterial community, but biomass management also was a significant source of variation. We were able to attribute variation in bacterial community composition to interactions between land management and time, and identified specific OTUs that were affected by such interactions. We also identified specific groups of OTUs that had differential enrichment under differing tillage and biomass treatments across time.

### **2.4.1 Soil and Environmental Factors**

Tillage significantly decreased soil C, soil N, and C:N (Table 2.1, soil C - F-value

= 77.2,  $p < 0.001$ ; soil N - F-value = 127.9,  $p < 0.001$ ; C:N- F-value = 14.7,  $p = 0.004$ ).

Plant biomass retention significantly increased soil C and soil N, but did not significantly affect C:N (soil C - F-value = 13.7,  $p = 0.005$ ; soil N - F-value = 7.7,  $p = 0.02$ ) . We observed a significant interaction of tillage with biomass retention for soil C (F-value = 6.4,  $p = 0.03$ ) but not for soil N or C:N. NTR plots had significantly higher soil C than NTH and till plots (Table 2.1, Pairwise and paired t-test,  $p < 0.05$ ), and NTH plots had higher soil C than till plots (Table 2.1, Pairwise and paired t-test,  $p < 0.05$ ) but till plots that varied in biomass removal did not significantly differ in soil C (Table 2.1, Pairwise and paired t-test,  $p \geq 0.05$ ). Soil N was significantly higher in both tilled treatments as compared to no-till treatments but within tillage, treatments did not differ significantly by biomass removal. Soil C:N ratios ranged from 11.2 -17.3, but were only significantly different for NTH and PTH treatments (Table 2.1, Pairwise and paired t-test,  $p = 0.05$ ). Soil temperature, moisture, and pH did not differ significantly among treatment plots (Supplementary Figure 2.2, Table 2.1).

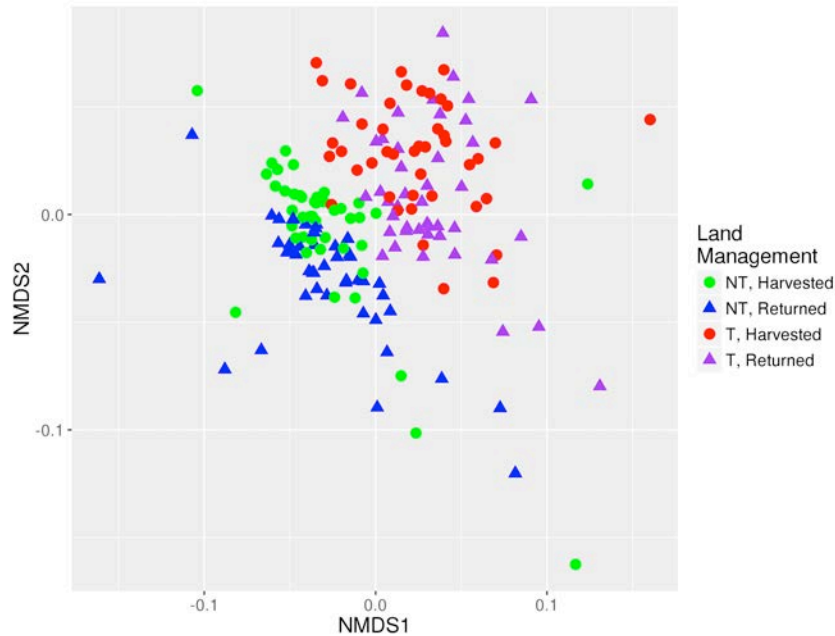
**Table 2.1.** Soil characteristics of the long term tillage experiment in Chazy, NY. All measurements, except moisture, were taken from soil in September 2014. Moisture was averaged over the 11 sampling timepoints from July 2014 – November 2015. Differing superscript letters in each column indicate significant differences between treatments.

Treatment	Tillage	Biomass Retention	%C	%N	C:N	pH	% Moisture
<b>NTR</b>	No	Yes	2.77 $\pm$ 0.48 <sup>a</sup>	0.23 $\pm$ 0.03 <sup>a</sup>	12.20 $\pm$ 0.68 <sup>ab</sup>	6.89 $\pm$ 0.68 <sup>a</sup>	17.1 $\pm$ 3.07 <sup>a</sup>
<b>NTH</b>	No	No	2.13 $\pm$ 0.39 <sup>b</sup>	0.18 $\pm$ 0.04 <sup>a</sup>	11.58 $\pm$ 0.32 <sup>a</sup>	6.89 $\pm$ 0.93 <sup>a</sup>	16.5 $\pm$ 2.47 <sup>a</sup>
<b>PTR</b>	Yes	Yes	1.62 $\pm$ 0.40 <sup>c</sup>	0.11 $\pm$ 0.01 <sup>b</sup>	14.36 $\pm$ 2.02 <sup>ab</sup>	7.66 $\pm$ 0.07 <sup>a</sup>	17.4 $\pm$ 8.19 <sup>a</sup>
<b>PTH</b>	Yes	No	1.50 $\pm$ 0.26 <sup>c</sup>	0.10 $\pm$ 0.02 <sup>b</sup>	14.36 $\pm$ 1.12 <sup>b</sup>	7.74 $\pm$ 0.05 <sup>a</sup>	16.5 $\pm$ 6.59 <sup>a</sup>



### ***2.4.2 Bacterial Community Composition Varies with Land Management and Time***

Non-metric multidimensional scaling (NMDS) ordination was used to visualize weighted UniFrac distances of SSU rRNA gene sequences. Bacterial communities vary by land management and date, with interaction between the two variables. More variation in bacterial community composition is explained by tillage (PERMANOVA, p-value = 0.001,  $F = 37.4$ ,  $R^2 = 0.18$ , Figure 2.1) than by biomass management (PERMANOVA, p-value 0.001,  $F = 6.7$ ,  $R^2 = 0.04$ , Figure 2.1). Altogether, land management (tillage and biomass management) accounts for 22.3% of variation in bacterial community composition (PERMANOVA, p-value 0.001,  $F = 16.6$ , Figure 2.1). Additionally, there is a significant interaction between tillage and biomass management which explains a small amount of variance in bacterial community composition (p-value = 0.018,  $R^2 = 0.01$ ,  $F = 2.3$ ). In contrast, temporal variation explains 15.8% of variation in bacterial community composition (PERMANOVA, p-value 0.001,  $F = 3.1$ , Supplementary Figure 2.3).



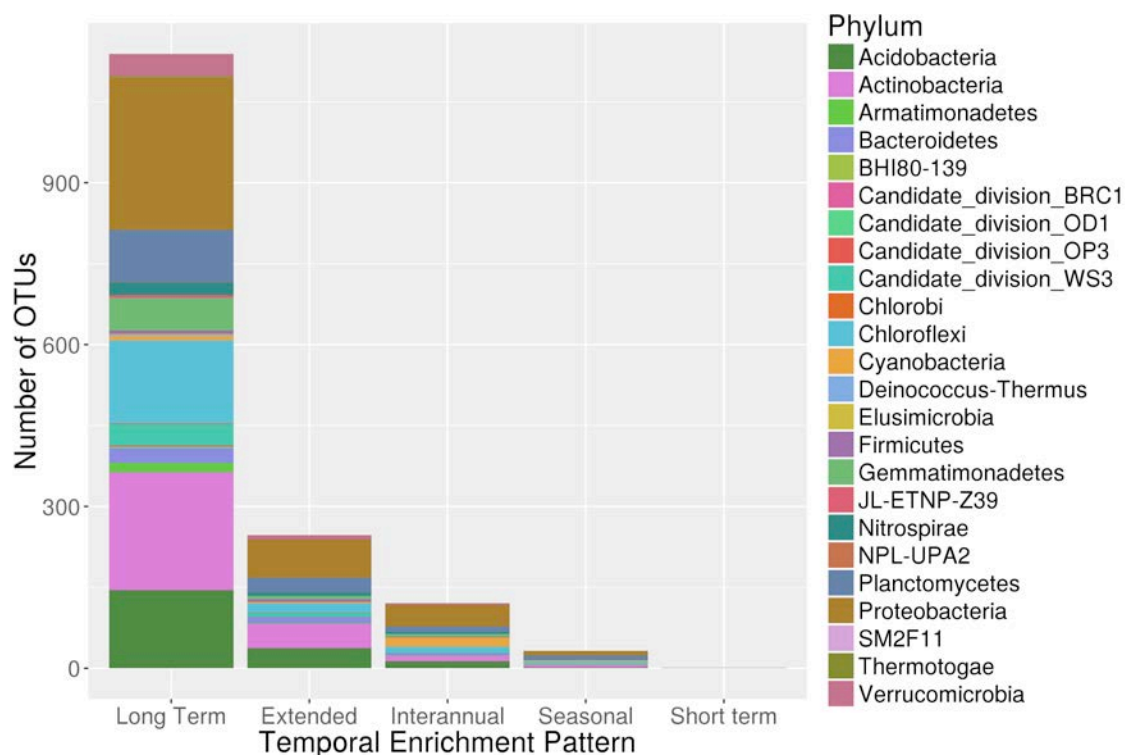
**Figure 2.1.** NMDS ordination of weighted Unifrac distances for each bacterial sample-time point combination. Colors indicate land management, with triangles representing organic matter returned and circles representing organic matter harvested

### ***2.4.3 Differential Enrichment of OTUs across Tillage and Time***

We found that enrichment of an OTU in either tillage condition over time was generally consistent (Smoothing spline ANOVA time series analysis, Figure 2.2). 1,513 of 3,964 (38%) OTUs were differentially enriched in tillage over time. OTUs that were differentially enriched with respect to tillage showed five different temporal patterns, as defined below. Long term enrichment (> 392 days of the 503 day sampling period) was observed across tillage for 1,138 OTUs. The long-term differentially enriched OTUs included 533 enriched in till and 606 enriched in no-till (Example OTUs, Supplemental Figure 2.4). Extended enrichment (210-392 days) was observed across tillage for 241 OTUs (93 till, 148 no-till, Supplemental Figure 2.5). Interannual enrichment (103 – 209 days) was observed across tillage for 84 OTUs (51 till, 33 no-till, Supplemental Figure

2.6). Seasonal enrichment (consistent pattern of enrichment with respect to growing season, 30 -120 days) was observed across tillage for 21 OTUs (12 till, 9 no-till, Supplemental Figure 2.7). Finally, episodic enrichment (one sampling period only, < 30 days) was observed across tillage for only one OTU (Supplemental Figure 2.8)

Phyla have different patterns of enrichment across tillage (Supplemental Figure 2.9). OTUs annotated as *Acidobacteria*, *Candidate division BRC1*, and *Elusimicrobia* have similar numbers of enriched OTUs in no-till soils as till soils. However, upon examination at closer taxonomic resolution (at the level of order), it is apparent that the *Acidobacteria* and *Elusimicrobia* – annotated OTUs differ in identity between treatments.



**Figure 2.2.** Number of OTUs enriched in either till or no-till conditions by temporal enrichment pattern. OTUs are colored by phylum annotation. Long term enrichment indicates enrichment for > 392 days of the 503 days during the sampling period. Extended enrichment indicates enrichment for 210 – 392 days, interannual enrichment is enrichment for 103 - 209 days, seasonal enrichment is for 30 -102 days, and short-term enrichment is less than 30 days.

In the majority of phyla, OTUs are unevenly enriched between the two soil conditions. *Actinobacteria*, *Candidate Division OD1*, *Candidate Division OP3*, *Candidate Division WS3*, *Chlorobi*, *Nitrospirae*, and *Verrucomicrobia* have higher numbers of OTUs enriched under no-till as compared to tilled conditions. Conversely, *Armatimonadetes*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, *Proteobacteria*, and *Thermotogae* have higher number of OTUs enriched under till as compared to no-till conditions.

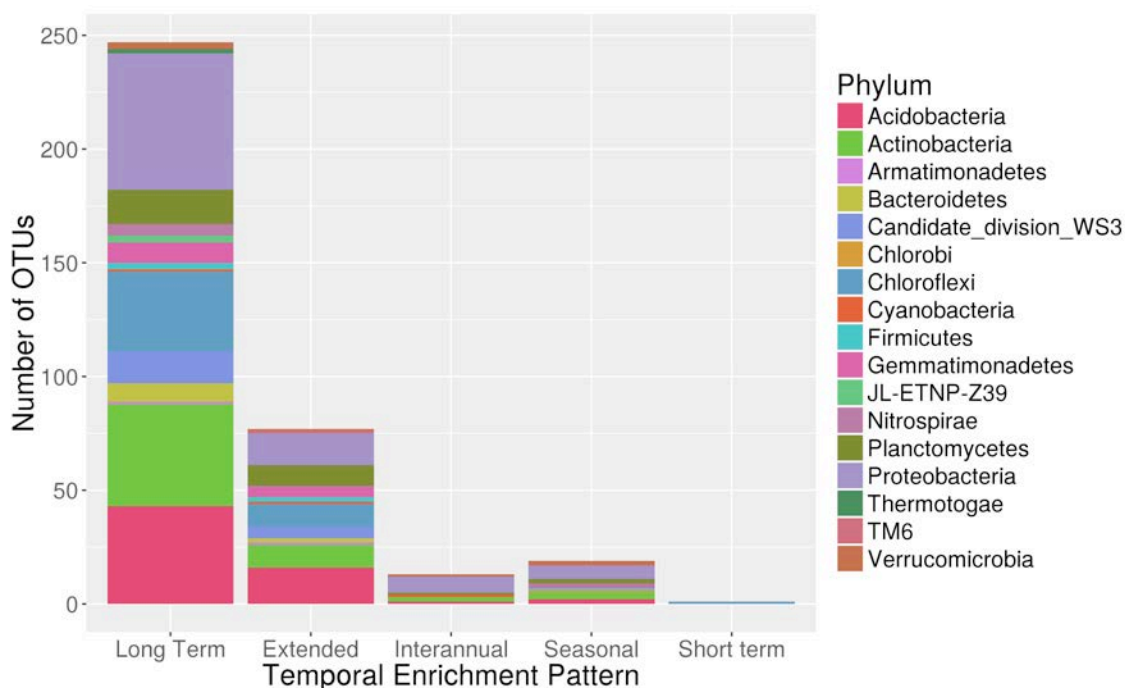
Within some of these phyla-level groups, the same orders are enriched under both no-till or till conditions (*Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes*, *Verrucomicrobia*). In others, though, distinct orders are enriched under no-till compared to till conditions (*Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Bacteroidetes*, *Chlorobi*, *Proteobacteria*).

#### **2.4.4 Differential Enrichment of OTUs Across Biomass Management and Time**

Biomass management impacts fewer OTUs than tillage, as 347 (8.7%) OTUs were differentially enriched in organic matter treatment over time. Similar to tillage, long term enrichment was seen for a majority (247) of OTUs (Figure 2.3). Long-term differentially enriched OTUs included 147 OTUs in biomass returned and 107 in biomass harvested conditions. Extended enrichment was seen for 77 OTUs (35 OTUs returned, 42 OTUs harvested). Interannual variation was observed for 13 OTUs (6 OTUs returned, 7 OTUs harvested), while seasonal enrichment was seen for 19 (9 OTUs returned, 10 OTUs harvested). Episodic response was present in 1 OTU

(enriched in harvested).

Similarly to the tillage treatment, differentially enriched OTUs belonging to the same phylum differ in number between harvested and returned treatment (Supplementary Figure 2.10). Only *Acidobacteria* and *Bacteroidetes* have similar numbers of enriched OTUs for the two treatments, but the OTUs in each treatment differ in composition at the order level.



**Figure 2.3.** Number of OTUs enriched in either biomass removed or biomass returned conditions by temporal enrichment pattern. OTUs are colored by phylum annotation. Long term enrichment indicates enrichment for > 392 days of the 503 days during the sampling period. Extended enrichment indicates enrichment for 210 – 392 days, interannual enrichment is enrichment for 103 - 209 days, seasonal enrichment is for 30 - 102 days, and short-term enrichment is less than 30 days.

Eleven phyla have more OTUs enriched in the harvested treatment and include *Actinobacteria*, *Armatimonadetes*, *Candidate Division WS3*, *Chlorflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, *JL-ETNP-Z39*, *Nitrospirae*, *Thermotogae*, and *TM6*.

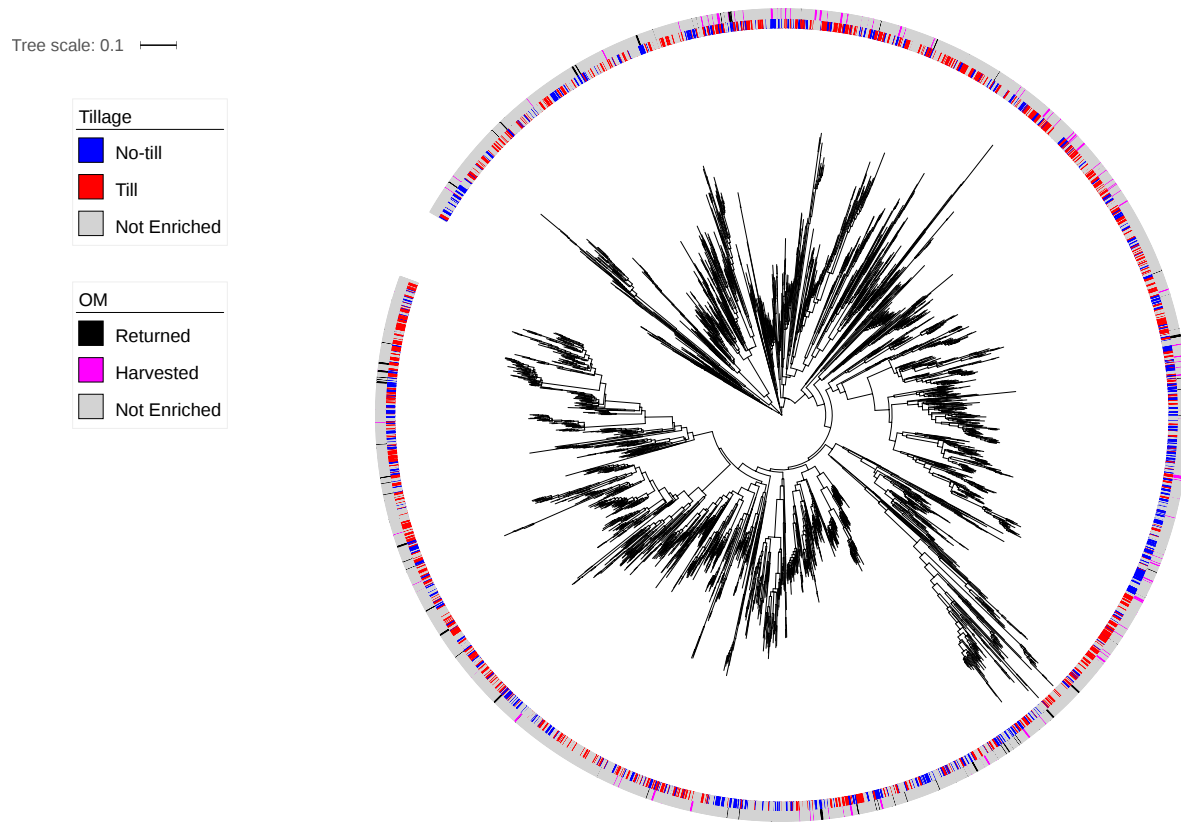
Three phyla have more OTUs enriched in the returned treatment, including *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia*.

#### **2.4.5 Response to Tillage and Biomass is Conserved Phylogenetically**

We tested phylogenetic conservation of enrichment response across treatments (NT vs T, R vs H) by calculating both net relatedness index (NRI) and nearest taxa index (NTI). NRI is a measure of the mean phylogenetic distance between all pairs of taxa that share a given trait in the community (Horner-Devine and Bohannan, 2006). NTI is a measure of the minimal branch length between all pairs of taxa that share a given trait in the community (Horner-Devine and Bohannan, 2006).

Communities of OTUs showing enrichment in specific tillage and biomass managements are more related phylogenetically than due to chance (Figure 2.4). Both communities of OTUs enriched in till and no-till managements have significant positive NTI values, but communities enriched in no-till management have significant positive NRI values while communities enriched in till management have non-significant negative NRI values (no-till: NRI = 4.67, NTI = 3.61; till: NTI = 3.57, NRI = -2.10). Communities enriched in biomass harvested and removed conditions both have significant and positive NRI and NTI values (harvested: NRI = 4.86, NTI = 3.36; returned: NRI = 2.05, NTI = 3.33). Thus, all treatment-specific communities are more phylogenetically related than due to chance, with no-till and biomass communities showing relationships between OTUs at both the tips and deeper within the phylogeny, while till communities are clustered at the tips but not necessarily at deeper

phylogenetic levels.

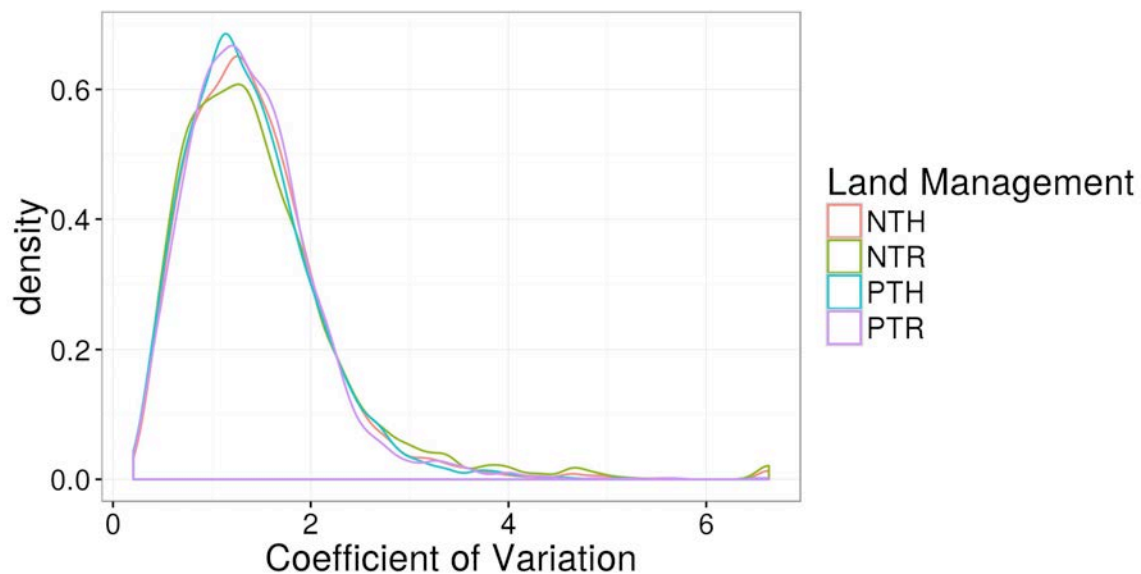


**Figure 2.4.** Phylogenetic tree of OTUs mapped with color strips indicating taxa enriched in differing tillage (inner circle) or organic matter management conditions (outer circle).

#### ***2.4.6 No-till, Returned Biomass OTUs are Highly Variable in Abundance Across Time***

To explore variation of OTUs over time, we calculated the coefficient of variation (CV) over time for the relative abundance of each OTU. The distribution does differ between treatments, with the NTR treatment differing from the others (Figure 2.5). The mean CV does not differ significantly across treatments (Kruskal Wallis,  $p = 0.08$ ; NTR :  $1.53 \pm 0.82$ , NTH :  $1.48 \pm 0.67$ , PTR :  $1.46 \pm 0.63$ , PTH :  $1.47 \pm 0.65$ ). However, the

skewness of the distribution, or the asymmetry of the distribution around the mean, is higher in NTR samples than till samples and positive for all treatments (NTR : 2.25, NTH : 1.77, PTR : 1.63, PTH : 1.39). Thus, OTUs within the NTR treatment have higher CVs or there are larger numbers of OTUs with higher CVs. When we subset to OTUs with a CV of 3.5 or greater, there are more OTUs in no-till than in till samples, especially in the NTR treatment (NTR : 110 OTUs, NTH : 65 OTUs, PTR : 42 OTUs, PTH : 40 OTUs). These OTUs come from *Chloroflexi* (43 OTUs), *Planctomycetes* (30 OTUs), *Acidobacteria* (29 OTUs), *Gemmatimonadetes* (24 OTUs), *Actinobacteria* (23 OTUs), *Betaproteobacteria* (21 OTUs), *Alphaproteobacteria* (19 OTUs), *Deltaproteobacteria* (14 OTUs), *Cyanobacteria* (14 OTUs), *Gammaproteobacteria* (11 OTUs), *Verrucomicrobia* (9 OTUs), *Bacteroidetes* (8 OTUs), *Candidate Division WS3* (4 OTUs), *Armatimonadetes* (3 OTUs), *Firmicutes* (2 OTUs), *JL-ETNP-Z39* (1 OTU), *Nitrospirae* (1 OTU), and *Thermotogae* (1 OTU) (Supplemental Figure 2.11).

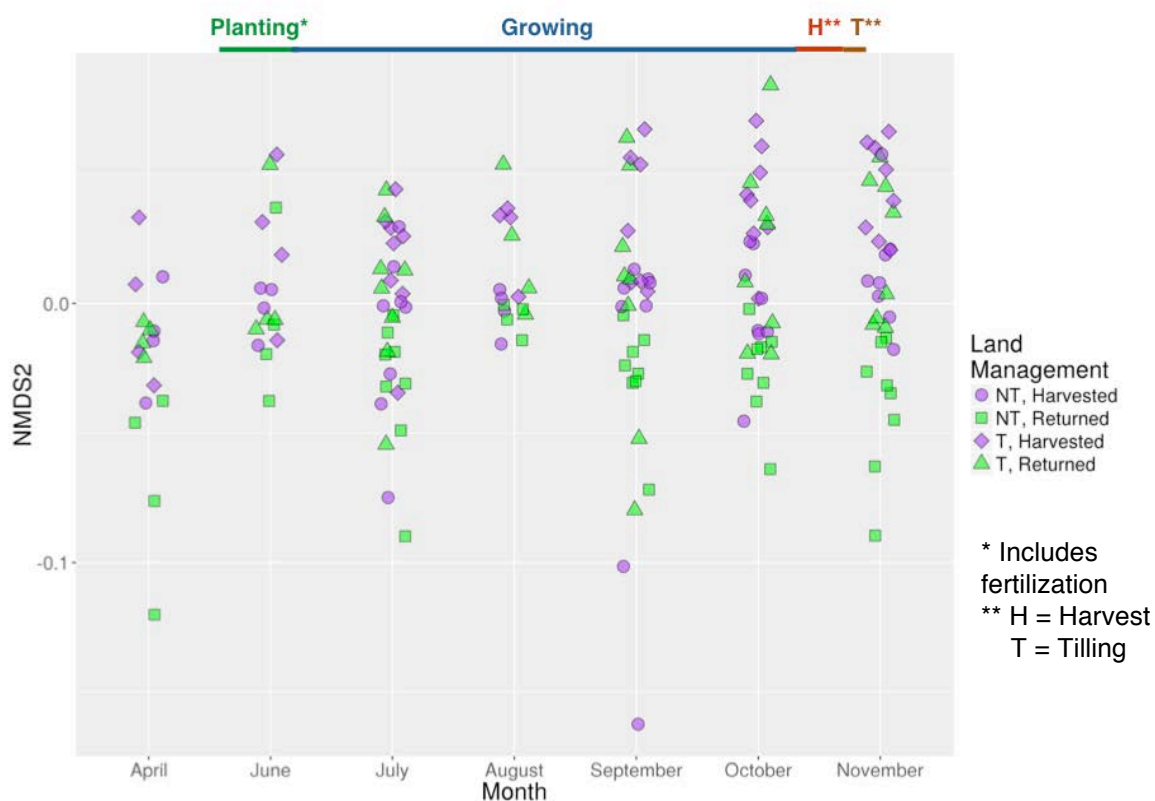


**Figure 2.5.** Kernel density distributions of the Coefficient of Variation (CV) of individual OTUs across time in each treatment. Colored lines demarcate treatments.



### 2.4.7 Interactions between Land Management and Time Contribute to Bacterial Community Composition Differences

Bacterial communities show a greater response to plant biomass retention in November and April, following the return of plant biomass to soil in November (Figure 2.6). In contrast, the effects of biomass retention are less apparent during the growing season from June through August (Figure 2.6). A significant interaction between biomass retention and time occurs within the no-till treatment (PERMANOVA,  $p$ -value = 0.042,  $R^2 = 0.12$ ,  $F = 1.3$ ), while no such interaction is observed within the tilled treatment (PERMANOVA,  $p$ -value = 0.91,  $R^2 = 0.08$ ,  $F = 0.83$ ).



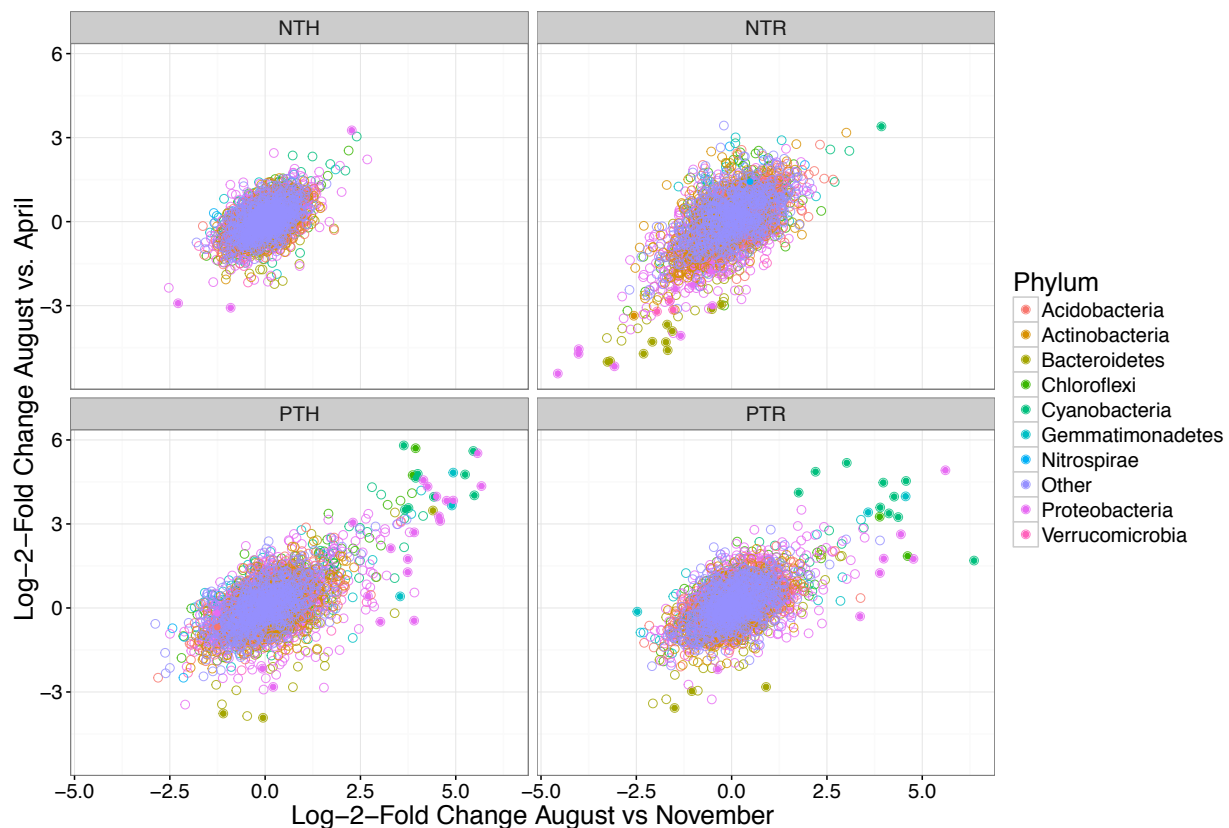
**Figure 2.6.** NMDS2 vs Month for individual samples, colored by organic matter treatment. Symbols indicate treatment status

#### **2.4.8 Identification of OTUs enriched in Samples from Spring, Summer, and Fall**

To further explore the interaction of biomass management with time, we quantified differential enrichment of OTUs between sampling dates in spring (April: pre-planting and fertilization), summer (August), and fall (November). These times were chosen because bacterial communities have increased variance attributed to biomass management in April and November (PERMANOVA; April :  $p = 0.04$ ,  $R^2 = 0.13$ ; November:  $p = 0.003$ ,  $R^2 = 0.10$  Figure 2.6), while biomass management does not contribute greatly to variance in bacterial communities in August (PERMANOVA,  $p = 0.5$ , Figure 2.6). Specifically, we found 25 OTUs that were enriched in NTR in November (6 OTUs, Figure 2.7, Supplemental Figure 2.12) or April (25 OTUs, Figure 2.7, Supplemental Figure 2.13), relative to August. These include OTUs that belong to *Proteobacteria* (orders: *Caulobacteriales*, *Sphingomonadales*, *Oxalobacteriales*, *Pseudomonadales*, and *Xanthomonadales*), *Bacteroidetes* (families: *Cytophagales*, *Flavobacteriales*, *Chitinophagales*, *Sphingobacteriales*), *Verrucomicrobia* (family: *Verrucomicrobiales*), and *Actinobacteria* (family: *Microbacteriales*).

The 25 OTUs that are differentially enriched over time in NTR show two different seasonal patterns in relation to the growing season. A total of 11 OTUs, including OTU.10 of the order *Flavobacteriales*, are enriched outside of the growing season. These OTUs have high relative abundance in April and November (with some OTUs also having high relative abundance in October) and reduced relative abundance in July – September (Supplemental Figure 2.14). The remaining 14 OTUs are enriched during the growing season. This pattern is represented by OTU.388 (order *Verrucomicrobiales*)

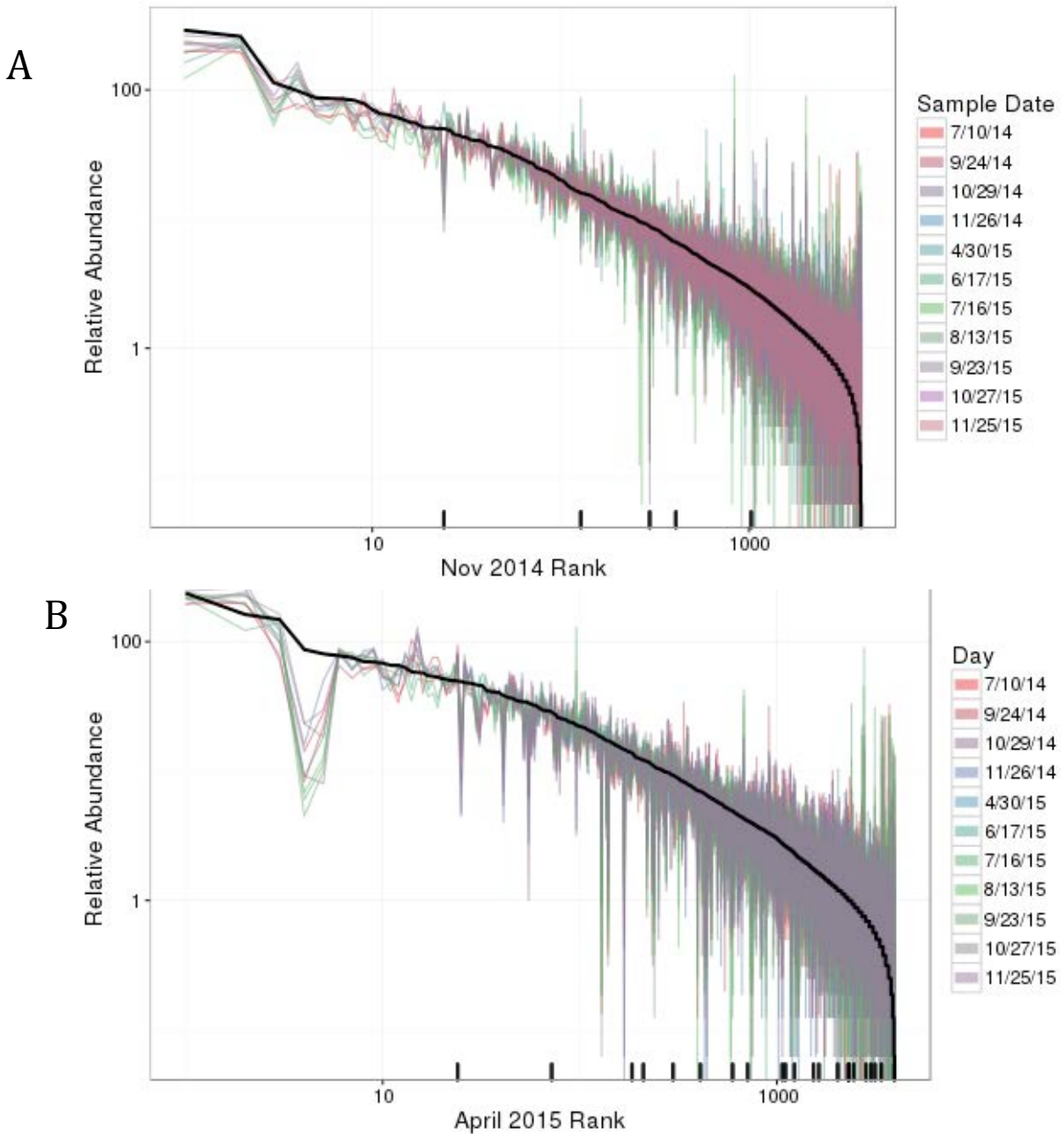
which is enriched in April, July, September, October, and November (Supplemental Figure 2.14). OTUs within the same family generally follow similar patterns of enrichment, with the exception of the *Sphingomonadaceae* (Supplemental Figures 2.15 – 2.18). Different temporal patterns for these OTUs are seen in the three other treatments (Supplementary Figures 2.19 – 2.21), suggesting that the temporal pattern seen in NTR samples is a specific interaction between time and this land management.



**Figure 2.7.** Plot comparing log<sub>2</sub>-fold change enrichment in August vs April against August vs November. Samples with negative x and y values indicate OTUs that are enriched in April and November, respectively, as compared to August. Positive x values indicate enrichment in August vs April and positive y-values indicate enrichment in August vs November. Filled circles are responders that are significantly differentially enriched in August, April, or November. Colors correspond to phylum annotation.

We also see 55 OTUs enriched in August as compared to April or November in the PTR and PTH treatments (Figure 2.7, Supplemental Figures 2.12, 2.13). These OTUs are classified as *Proteobacteria* (24 OTUs, families *Caulobacteraceae*, *Comamondadaceae*, *Erythrobacteraceae*, *Rhodobacteraceae*, *Rhodocyclaceae*, *Sphingomonadaceae*, *Xanthamonadaceae*, and unknown), *Cyanobacteria* (20 OTUs, families *Leptolyngbya*, *Microcoleus*, *Phormidium*, and unknown), *Gemmatimonadetes* (5 OTUs, families *Gemmatimonas* and unknown), *Chloroflexi* (4 OTUs, family *Chloroflexus*), *Bacteroidetes* (1 OTUs, family *Cytophagaceae*), and *Nitrospirae* (1 OTU, family *0319-6A21*).

Seasonally responsive OTUs changed in abundance dramatically over time (Figure 2.8). We quantified the relationship between temporal variability and relative abundance by calculating the coefficient of variation of seasonally responsive OTUs over time. OTUs identified as differentially enriched in the fall NTR treatment had the highest mean CV ( $2.2 \pm 0.4$ ), followed by PTR and PTH summer responders ( $2.0 \pm 0.8$ ;  $1.8 \pm 0.4$ ). NTR spring responders, though numerous, had lower CV values (mean :  $1.6 \pm 0.9$ ).



**Figure 2.8.** Bulk rank abundance curves for **(A)** November 2014 sampling time point and **(B)** April 2015 sampling time point. Abundance of other time points is indicated by colored lines, but rank of OTUs is determined by November or April data. Rank positions of OTUs enriched in NTR treatment in either November **(A)** or April **(B)** vs. August are indicated by rug at the bottom of the plot.

## 2.5 Discussion

We were able to partition variance in soil bacterial communities from the same soil type but differing land management strategies to several sources. The largest source of variance identified was sampling date, followed by tillage and biomass management. Interactions between time and land management also contributed to variation in bacterial communities. Different OTUs had different responses with time and land management : some showed consistent enrichment over time in a certain land management, but others showing variation in enrichment over time, depending on land management.

Date or seasonality previously has been shown to be the largest contributor to variation in soil bacterial communities, which is consistent with our findings (Birgander et al., 2014; Buckley and Schmidt, 2003; Spedding et al., 2004; Stevenson et al., 2014). Treatments showed differing distribution of variation of relative abundance of individual OTUs over time, with NTR treatments having more OTUs that varied highly in abundance over time. OTUs that are differentially enriched across seasons (fall, spring, or summer) in the NTR treatment have a broader distribution of CV values than in other treatments.

Evidence for long-term impact of land management on OTU enrichment was identified. We find that the majority of OTUs that are enriched in one form of tillage or one form of biomass management tended to remain enriched over the entire course of sampling. Studies looking at seasonal effects on soils under varying fertilizer treatments found similar results, with certain bacterial groups affected by treatment more than

seasonal variation (Bardgett et al., 1999; Jangid et al., 2008). When phylogenetic conservation of these long-term groups is explored, evidence suggests that the OTUs enriched in the same treatment are more genetically similar than would be seen by chance. These OTUs may have life history strategies that contribute to their uneven representation under certain tillage or biomass management conditions.

Some OTUs did show dynamic enrichment over time in terms of tillage and biomass management. OTUs varied in enrichment inter-annually, with OTUs enriched in one treatment for extended spans in 2014 or 2015, but not both. Additionally, several OTUs were enriched for multiple, discontinuous timespans during the sampling period, consistent with seasonal enrichment that we see in the NTR treatment.

Tillage and biomass management are also known to affect bacterial community composition, as seen in other studies using long-term field sites (Babujia et al., 2010; Jiménez-Bueno et al., 2016; Navarro-Noya et al., 2013; Ramirez-Villanueva et al., 2015). We confirmed similar phyla that are differentially enriched across tillage, such as enrichment of OTUs from *Actinobacteria* in the no-till treatment and enrichment of OTUs from *Planctomycetes*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, and *Proteobacteria* in tillage management, as seen in previous studies (Jiménez-Bueno et al., 2016; Navarro-Noya et al., 2013). Similarly, we saw increased numbers of *Actinobacteria*, *Gemmatimonadetes*, and *Chloroflexi* OTUs enriched under harvested biomass conditions, while *Bacteroidetes*, *Proteobacteria*, *Planctomycetes* and *Acidobacteria* were favored under returned biomass management, similar to previous studies (Jiménez-Bueno et al., 2016; Navarro-Noya et al., 2013; Ramirez-Villanueva et

al., 2015).

We also show that temporal variation in bacterial community composition in response to biomass retention is greater in no till soils than in tilled soils. Tillage can have a large effect on soil structure, chemistry, and biology. Tilled soils have a reduced level of aggregation as compared to no-till soils (Govaerts et al., 2009; John et al., 2005), and often have increase compaction and erosion (Govaerts et al., 2009). Due to these differences in soil structure, till soils generally have reduced moisture and reduced carbon and nitrogen stocks (Table 2.1, Davidson and Ackerman, 1993) and may contain different microbial habitats as compared to no-till soils. The combination of differences in soil structure and soil chemistry may contribute to the variation we see in the bacterial communities of biomass returned treatments across tillage and time.

Additionally, interactions between microbial communities and carbon from plant roots and/or plant biomass returned to the plot may be implicated. Microbial communities from NTR plots differ in composition from NTH plots during the end of the growing season and during the non-growing season (April, October, November), but become more similar during the peak of the growing season (June – August). This corresponds with the return of plant biomass in biomass returned treatments after crop harvest in early November.

During the early growing season (June – August), the corn plants are producing root exudates that can increase the carbon content of the surrounding soils (Wardlaw, 1990). The introduction of this labile carbon may contribute to homogenization of communities in all treatments. Root exudation can strongly affect microbial community



activity and composition (Pascault et al., 2013). Respiration (normalized by biomass) measured from soils under till and no-till management responds to rhizodeposition in similar ways (Franzluebbers et al., 1995). Thus, the presence of root exudates and dead roots may contribute to more similar communities during the growing season, regardless of land management

During the end of the growing season (September, October), the corn shifts carbon resources to grain production and reduces root exudates (Wardlaw, 1990). In the non-growing season (November – May), corn plants have been removed from the soil and the only carbon sources are the corn stover returned to only the biomass returned plots and carbon contained in the soil. The increased carbon content in the NTR soil, may support a different community as compared to the other land managements. Carbon availability can drive seasonal changes in microbial community, with reduction of plant root exudation in autumn proposed as a driving factor for community change in the fall (Griffiths et al., 2003; Stevenson et al., 2014; Thoms and Gleixner, 2013). Previous work has also found that addition of plant biomass to soils results in bacterial community changes, compared to soils not receiving stover amendment (Whitman et al., 2016). Other studies looking at alfalfa or wheat biomass addition to agricultural soils and litter amendments on forest soils have also found that addition of carbon to soils affects bacterial communities (Pascault et al., 2013; Pfeiffer et al., 2013).

The PTR treatment also has biomass applied in early November but the biomass is then immediately tilled into the soil. As the bacterial community in the PTR treatment

does not vary as much seasonally as the NTR treatment, we can hypothesize that tillage may reduce or negate the seasonal effect of biomass application on bacterial community structure.

With our sequencing data, we are able to identify individual OTUs that are significantly enriched in the fall and spring (when the NTR community separates from the other communities) as compared to the summer (when the communities of all four treatments are similar). We identified 24 such OTUs, from the phyla *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Verrucomicrobia*. The OTUs identified as enriched in NTR in fall or spring have similar taxonomic identity to OTUs identified in study by Whitman et al. (2016) as enriched in response to the addition of either maize biomass or pyrogenic organic matter (PyOM) on an agricultural soil. The addition of maize biomass caused significant enrichment of OTUs from the orders *Cytophagales* (*Bacteroidetes*), *Flavobacteriales* (*Bacteroidetes*), *Sphingobacteriales* (*Bacteroidetes*), *Sphingomonadales* (*Proteobacteria*), *Xanthomonadales* (*Proteobacteria*), and *Pseudomonadales* (*Proteobacteria*). *Verrucomicrobiales* responded significantly to both stover and PyOM treatment, and *Oxalobacterales* (*Proteobacteria*) responded to PyOM treatment only (Whitman et al., 2016). These orders are representative of nearly all the differentially enriched OTUs in the NTR treatment in spring or fall vs. summer in this study. This suggests that these OTUs respond to addition of biomass. The Whitman et al. study found that the response of OTUs to maize biomass addition was transient and declined after 82 days. A similar temporal phenomenon may explain why the interaction between biomass and time disappears by summer in this experiment.

Additionally *Flavobacterium* have previously been shown to be preferentially enriched in no-till and biomass-returned land managements (Navarro-Noya et al., 2013). In stable isotope probing studies looking at biomass addition and decomposition, *Beta-proteobacteria* and *Bacteroidetes*, are enriched in decomposition-associated samples (Bernard et al., 2007; Padmanabhan et al., 2003; Pascault et al., 2013; Pepe-Ranney et al., 2016). SSU rRNA of *Beta-proteobacteria*, specifically *Oxalobacteriales* was found to be enriched in samples with leaf litter addition compared to samples that did not receive litter (Pfeiffer et al., 2013). These are all groups of bacteria that we find enriched in the fall or spring in the NTR treatment, providing evidence for their increase in abundance due to growth on returned corn biomass.

Most of the OTUs identified as differentially enriched in the NTR treatment for spring – summer and fall – summer comparisons are enriched in the spring. Biomass was placed on the soil in early November, and we sampled soils in late November. Thus, the microbial community may need time to respond to biomass placed on soil or for that change to be represented in the soil DNA pool. The five OTUs that did respond in the November vs. August comparison increased in relative abundance about 32-fold, with some at high abundances in the community rank abundance curve in November (Supplementary Figure 6). Their increase in abundance may have been enough to result in community level differences. As we did not sample from November – April, we are unable to comment on the community dynamics and differential enrichment of OTUs during those time points, but evidence exists for impact of biomass application on microbial community through April of the year following biomass application.

## 2.6 Conclusion

This study shows that sources of variance in bacterial communities can be complex, even in soils of the same soil type and location that differ only in land management. We found that season, tillage, and biomass management affect composition of bacterial communities. However, interactions between season and land management can also be sources of variance. We found examples of OTUs that remained consistently enriched in certain land management conditions regardless of time, and OTUs that varied in enrichment over season. Specifically, OTUs in bacterial communities under NTR management varied in enrichment over time. We hypothesize that carbon supply to the bacterial communities, altering from root exudates during the growing season and returned crop biomass in the non-growing season contributes to such community dynamics. Further understanding of interactions of seasonal dynamics with land management or other factors may provide richer understanding of soil bacterial community dynamics and the role of those dynamics in relation to the soil carbon cycle.

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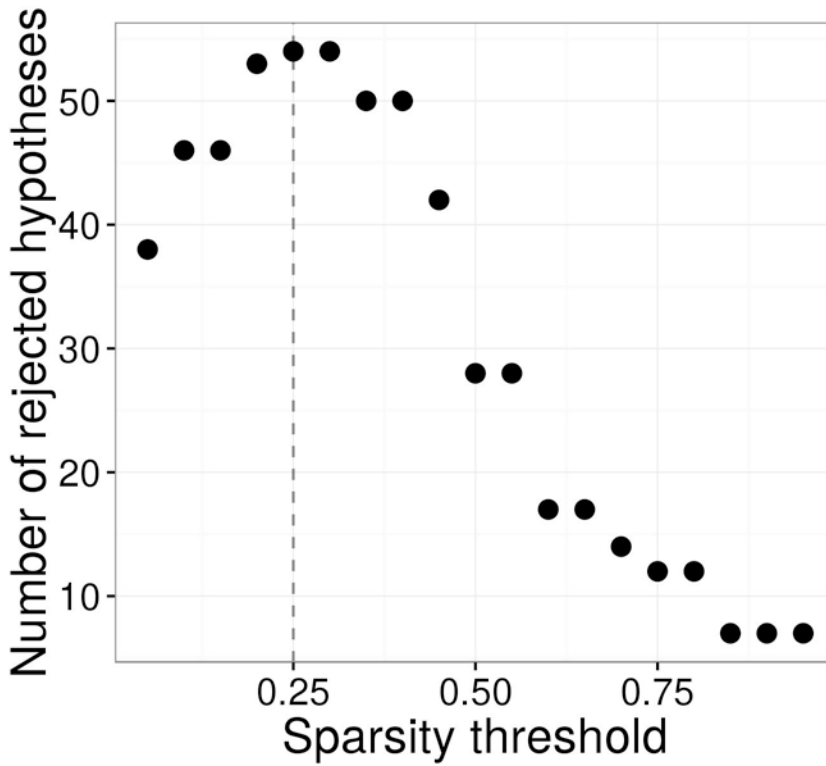
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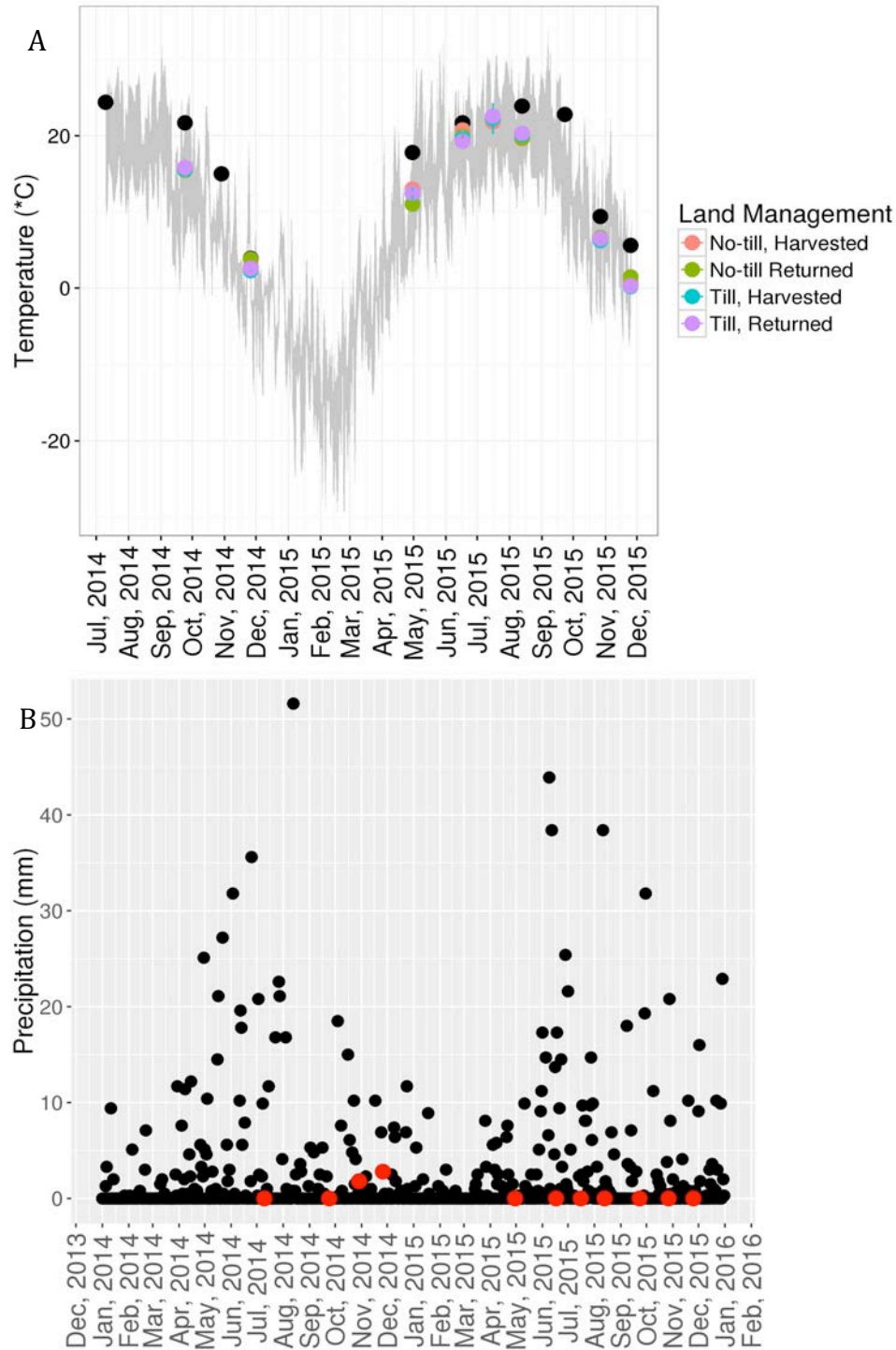
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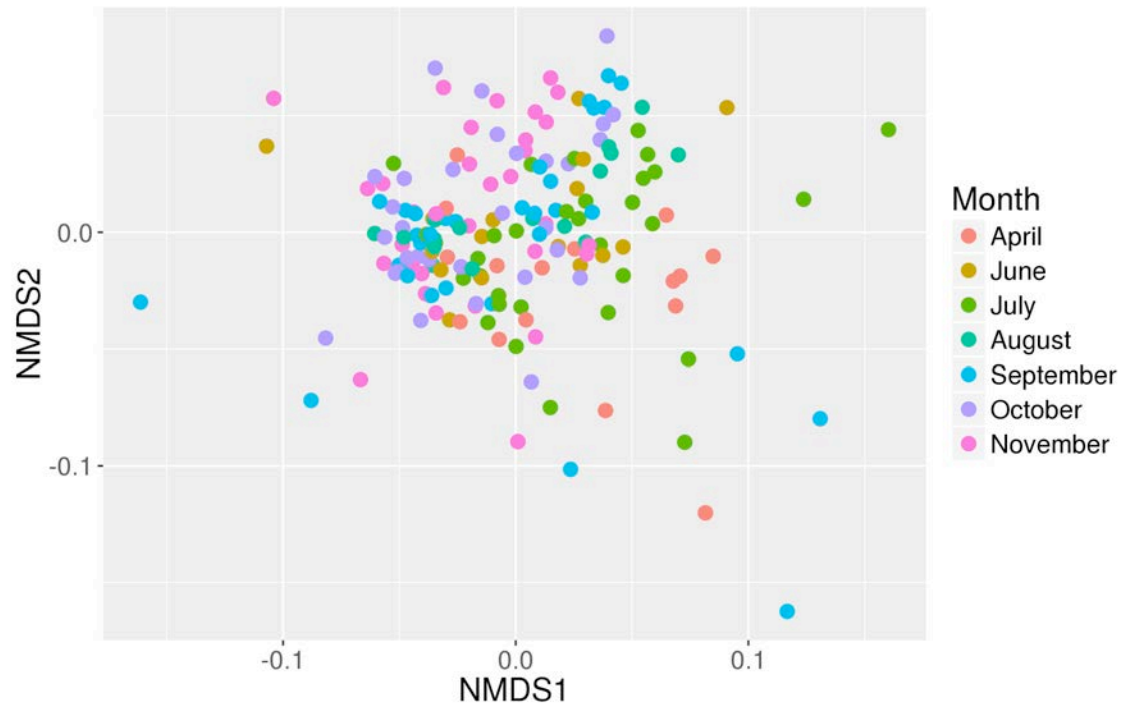
## 2.8 Supplemental Figures



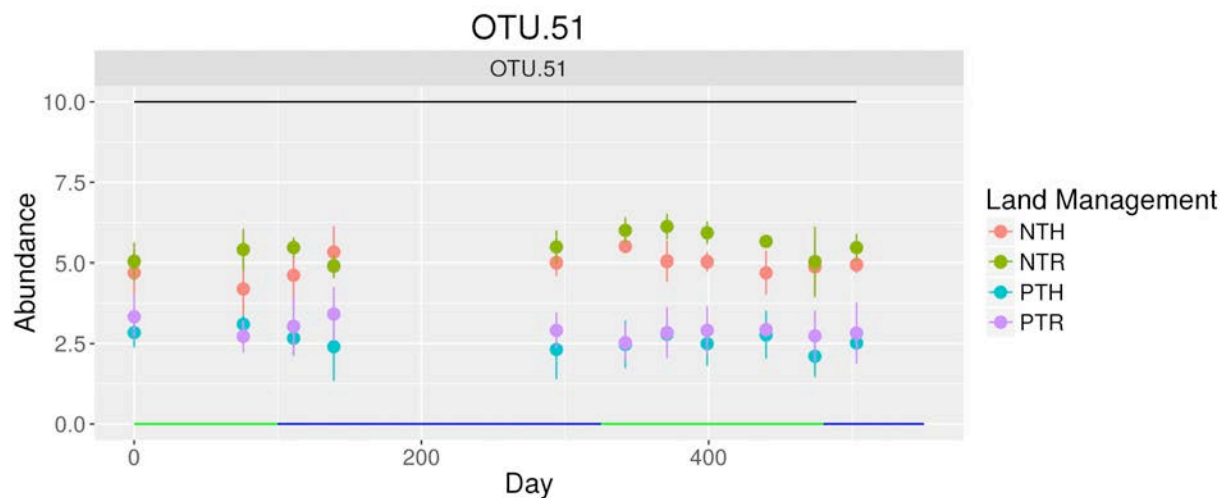
**Supplemental Figure 2.1.** Number of rejected hypothesis ( $p < 0.10$ ) for  $\log_2$ -fold change values comparing of OTU abundance across conditions (seasonal comparisons, tillage comparison, and biomass comparison) at each sparsity threshold. The sparsity threshold with higher number of rejected hypotheses (0.25) was chosen for independent filtering of results.



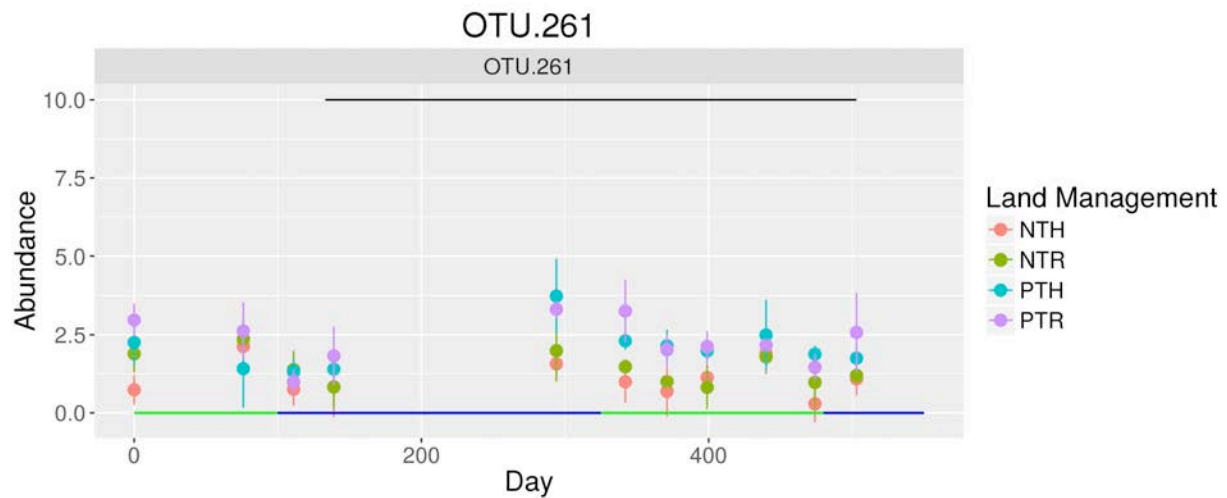
**Supplemental Figure 2.2. (A)** Ambient air temperature range over sampling time period. Black points represent maximum air temperatures on sampling days. Colored points represent soil temperatures for each land management on subset of sampling days (July 2014, October 2014, and September 2015 excluded) **(B)** Precipitation values (mm) for the sampling time period. Red dots indicate precipitation levels on sampling days.



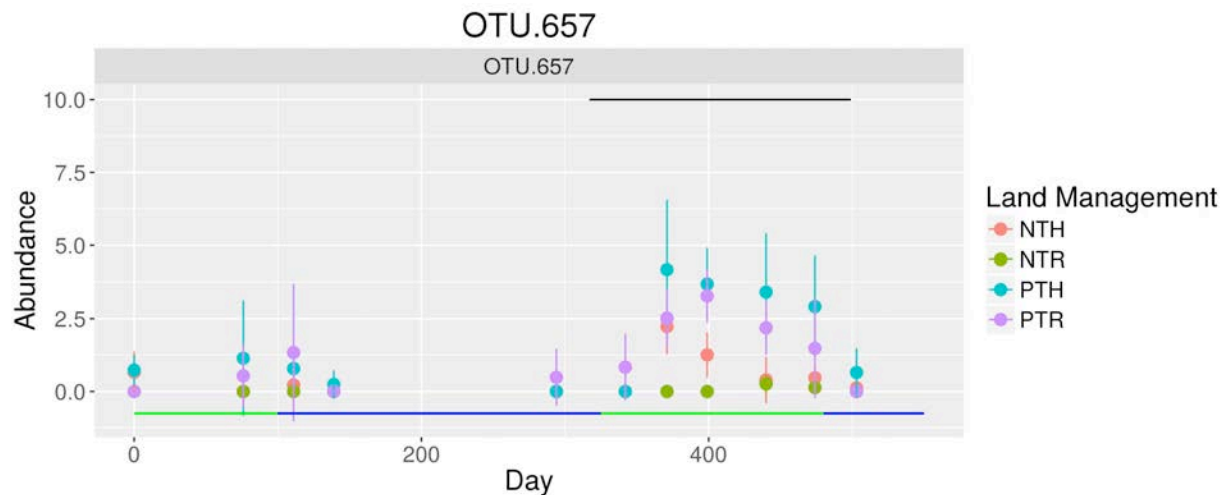
**Supplemental Figure 2.3.** NMDS ordination of Weighted Unifrac distances for SSU rRNA sequences in each bacterial sample-time point. Colors indicate month of sampling.



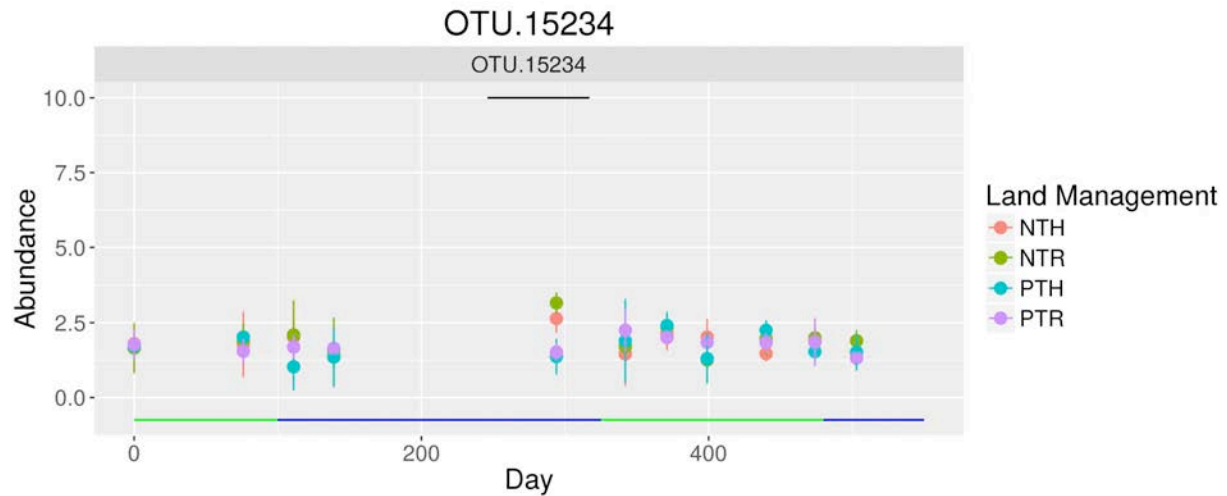
**Supplemental Figure 2.4.** Example of OTU significantly differentially enriched across tillage in the ‘long term’ category ( > 392 consecutive days of 503 days that encompass the sampling timepoints). The top black bar represents periods where samples have significantly different abundances across tillage. Points are colored by land management. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.



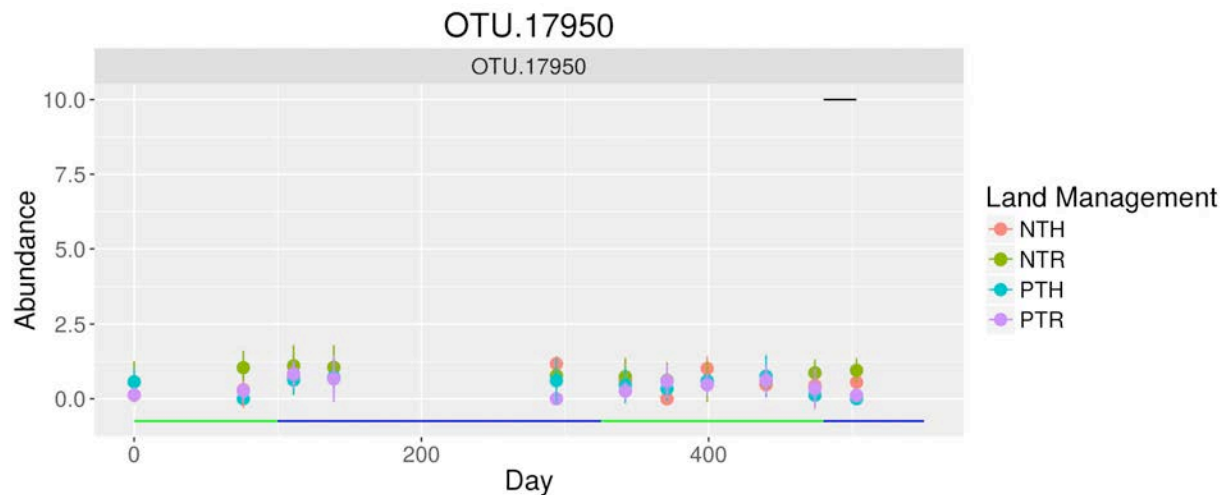
**Supplemental Figure 2.5.** Example of OTU significantly differentially enriched in the 'Extended' category (210-392 consecutive days of 503 days that encompass the sampling timepoints). The top black bar represents periods where samples have significantly different abundances across tillage. Points are colored by land management. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.



**Supplemental Figure 2.6.** Example of OTU significantly differentially enriched in the 'Interannual' category (103-209 consecutive days of 503 days that encompass the sampling timepoints). The top black bar represents periods where samples have significantly different abundances across tillage. Points are colored by land management. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.



**Supplemental Figure 2.7.** Example of OTU significantly differentially enriched in the 'Seasonal' category (30 -102 consecutive days of 503 days that encompass the sampling timepoints). The top black bar represents periods where samples have significantly different abundances across tillage. Points are colored by land management. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.

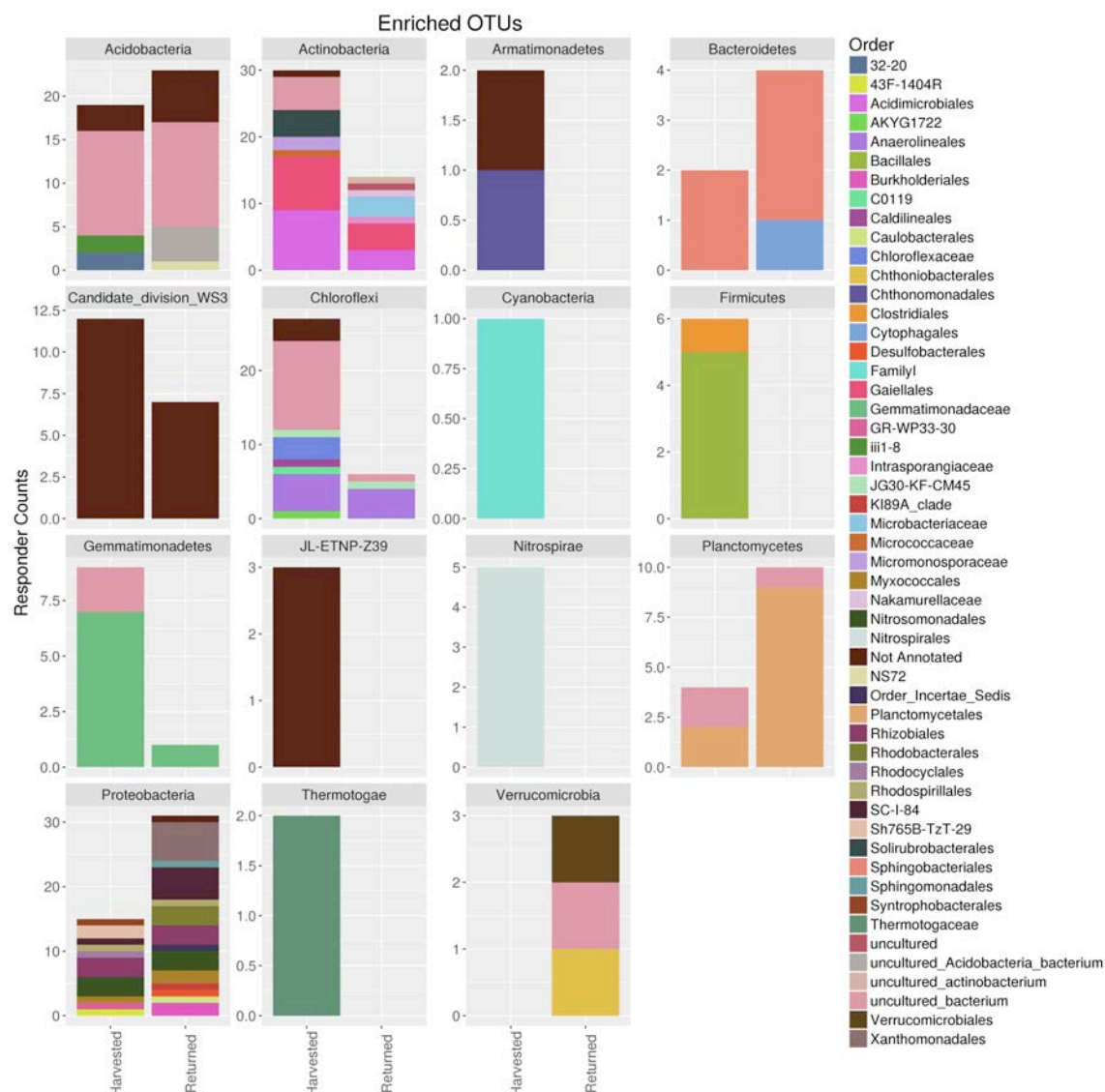


**Supplemental Figure 2.8.** Example of OTU significantly differentially enriched in the 'Episodic' category (< 30 consecutive days of 503 days that encompass the sampling timepoints). The top black bar represents periods where samples have significantly different abundances across tillage. Points are colored by land management. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.

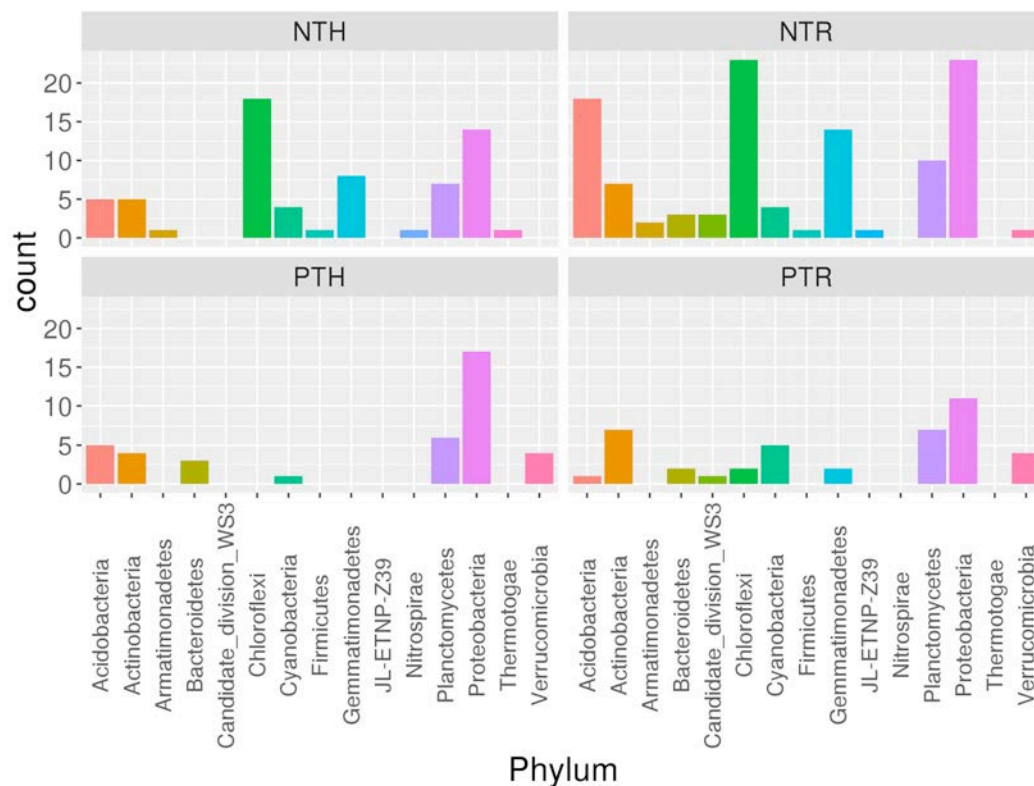




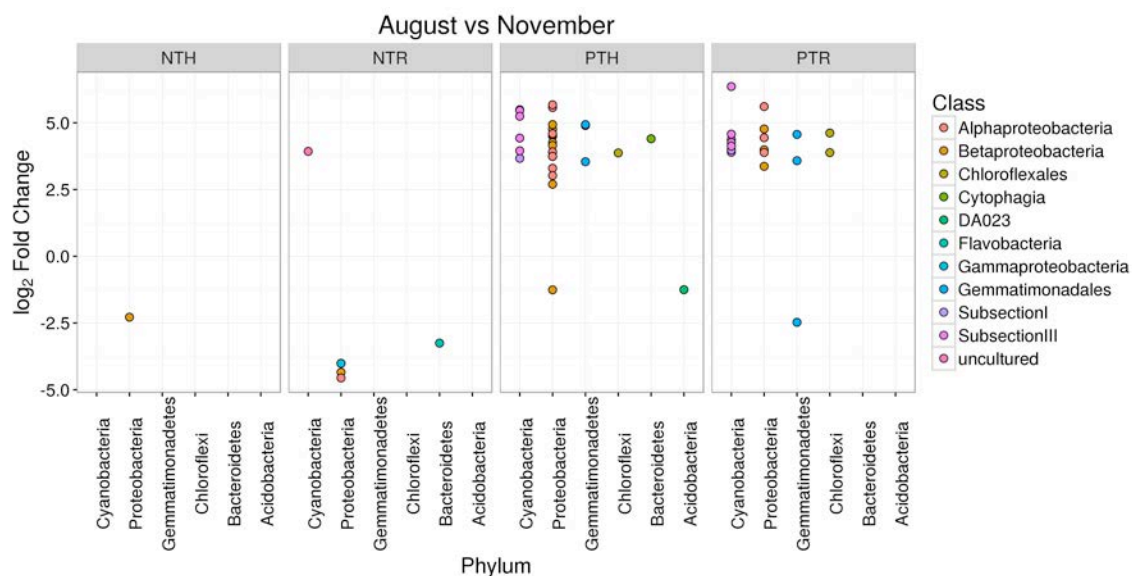
**Supplementary Figure 2.9.** Counts of OTUs enriched in each tillage condition. OTUs are faceted by phylum and colored by order classification.



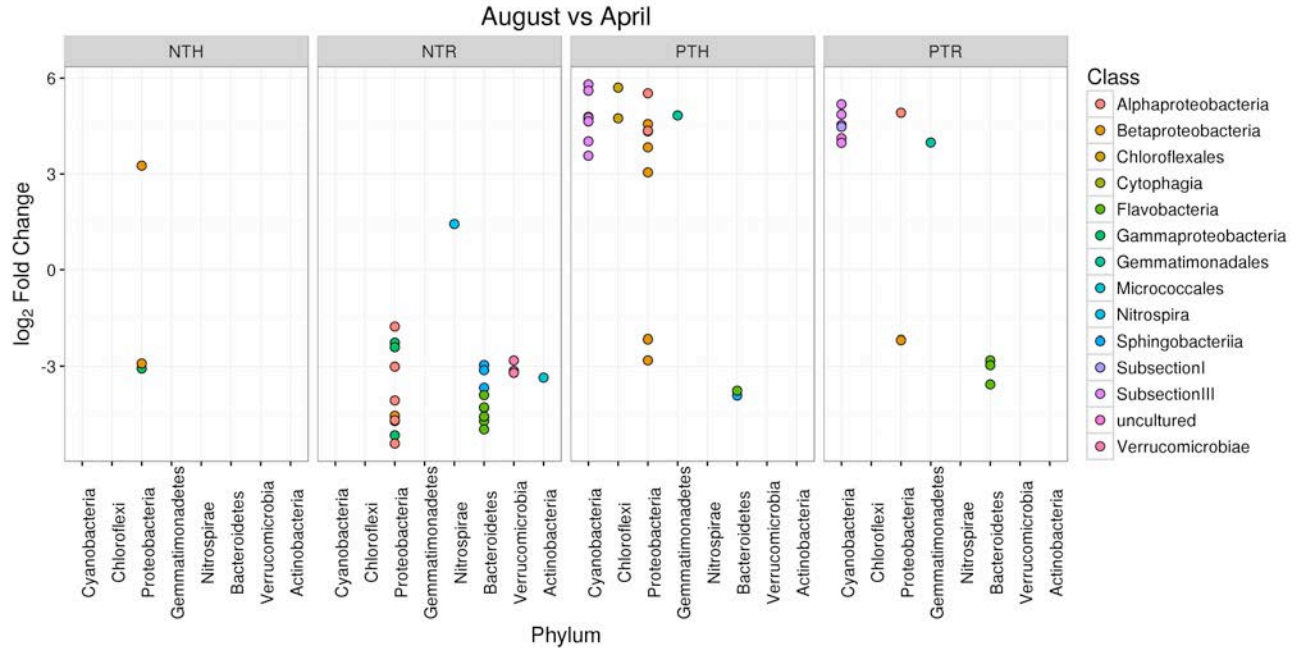
**Supplementary Figure 2.10.** Counts of OTUs enriched in each biomass management condition. OTUs are faceted by phylum and colored by order classification.



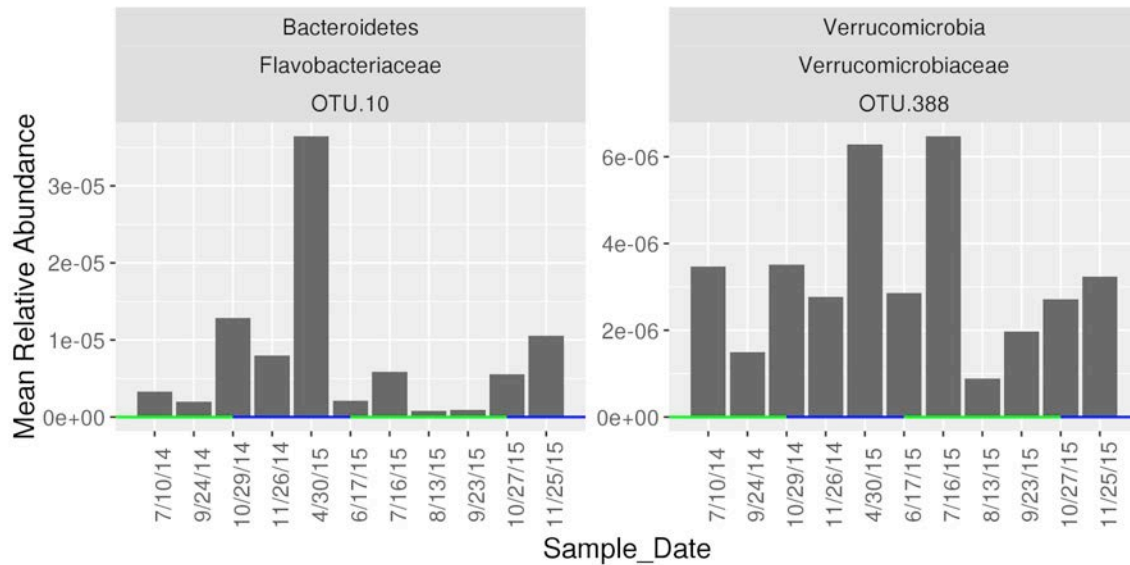
**Supplemental Figure 2.11.** Counts of OTUs in each phylum with coefficient of variation greater than 3.5, faceted for each treatment. Color indicate phylum annotation.



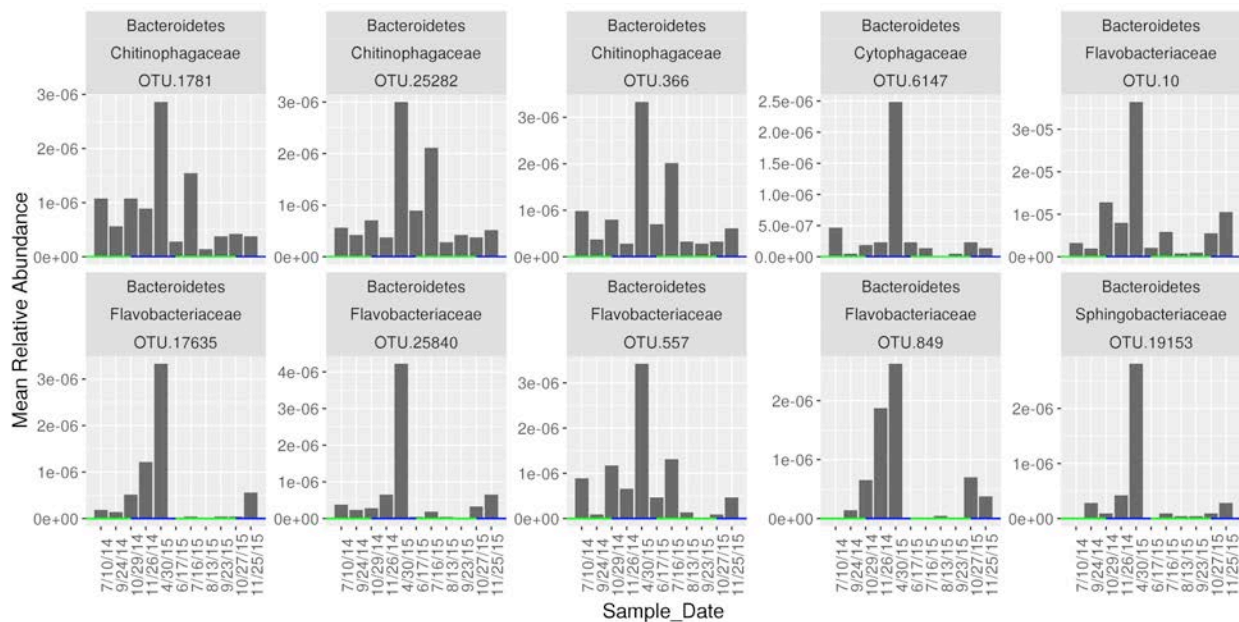
**Supplemental Figure 2.12.** Log<sub>2</sub>-fold change of OTU abundance in August vs November. Only significantly differentially enriched OTUs (Wald test,  $p < 0.10$ ) are shown. OTUs are ordered and colored by phylum and faceted by land management condition. A negative log<sub>2</sub>-fold change value indicates enrichment of OTU in November, while positive log<sub>2</sub>-fold change values indicate enrichment in August.



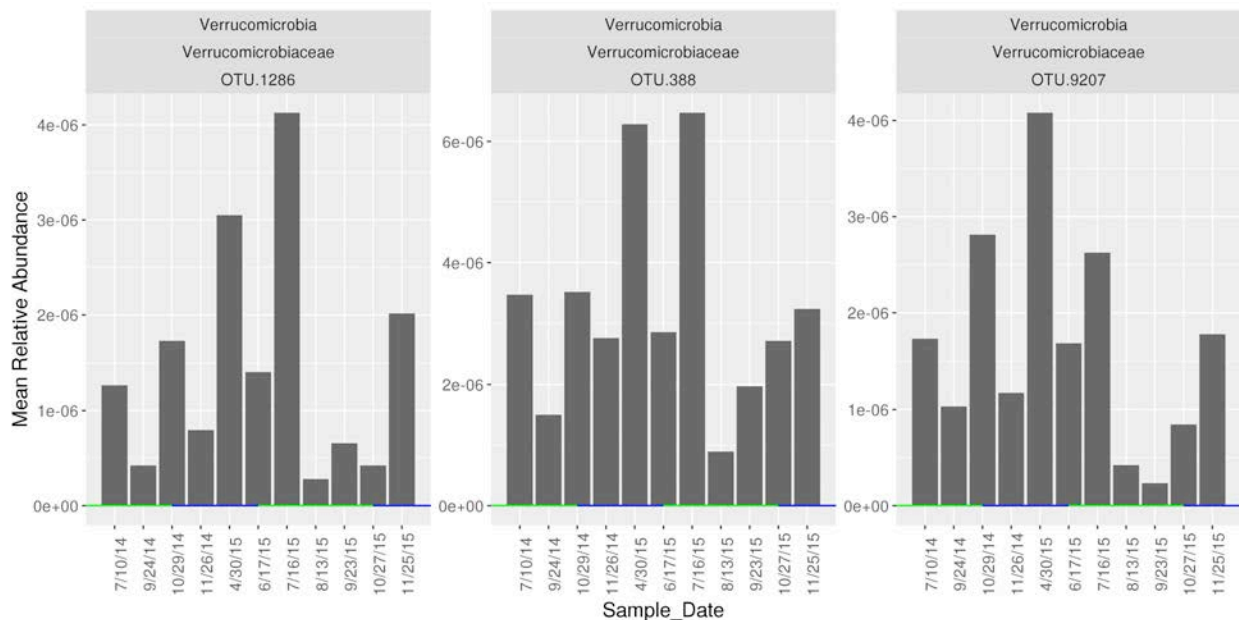
**Supplemental Figure 2.13.** Log<sub>2</sub>-Fold Change of OTU abundance in April vs August. OTUs are ordered and colored by phylum and faceted by land management condition. Only significantly differentially enriched OTUs (Wald test,  $p < 0.10$ ) are shown. A negative log<sub>2</sub>-fold change value indicates enrichment of OTU in April, while positive log<sub>2</sub>-fold change values indicate enrichment in August.



**Supplemental Figure 2.14.** Two main patterns of relative abundance over time for OTUs in NTR treatment enriched in November and April. Mean relative abundance for representative OTU.25 and OTU.388 over sampling time points. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.

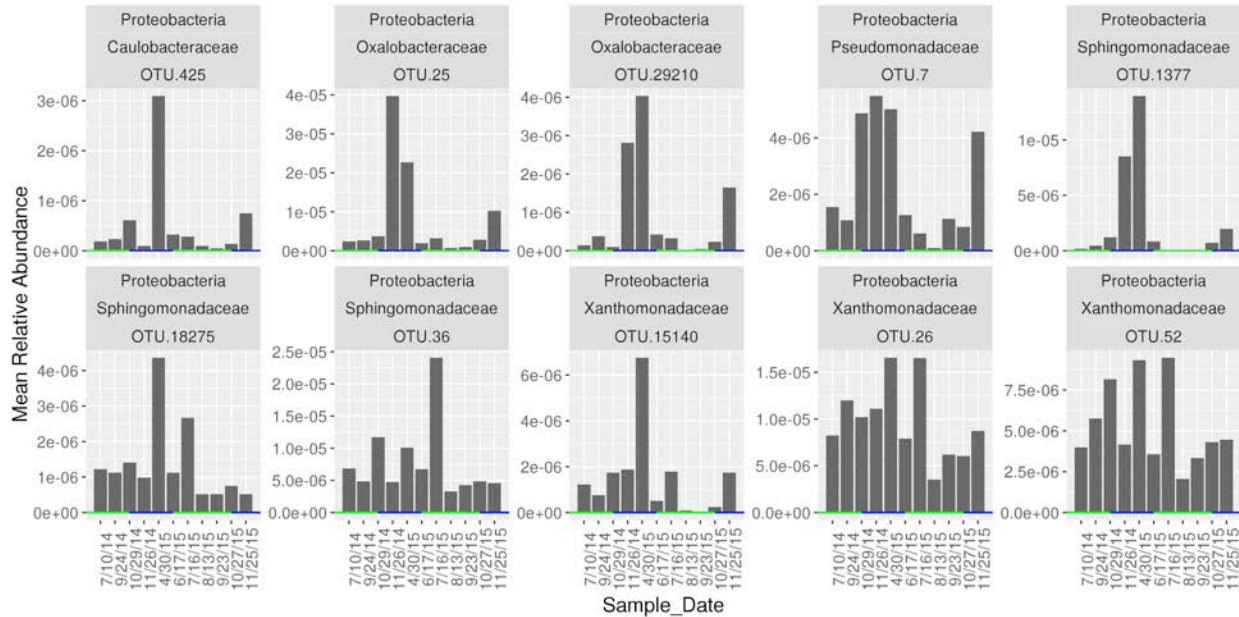


**Supplemental Figure 2.15.** Mean relative abundance over sampling time points in the NTR treatment of *Bacteroidetes* OTUs identified as enriched in either April or November, as compared to August. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.

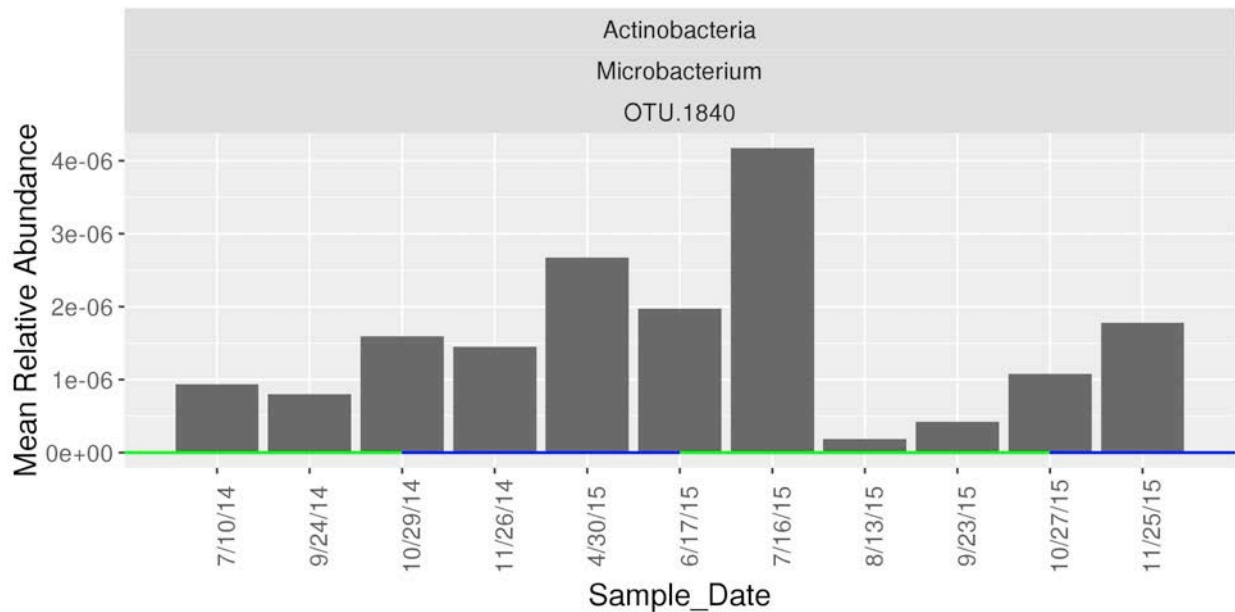


**Supplemental Figure 2.16.** Mean relative abundance over sampling time points in the NTR treatment of *Verrucomicrobia* OTUs identified as enriched in either April or November, as compared to August. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.

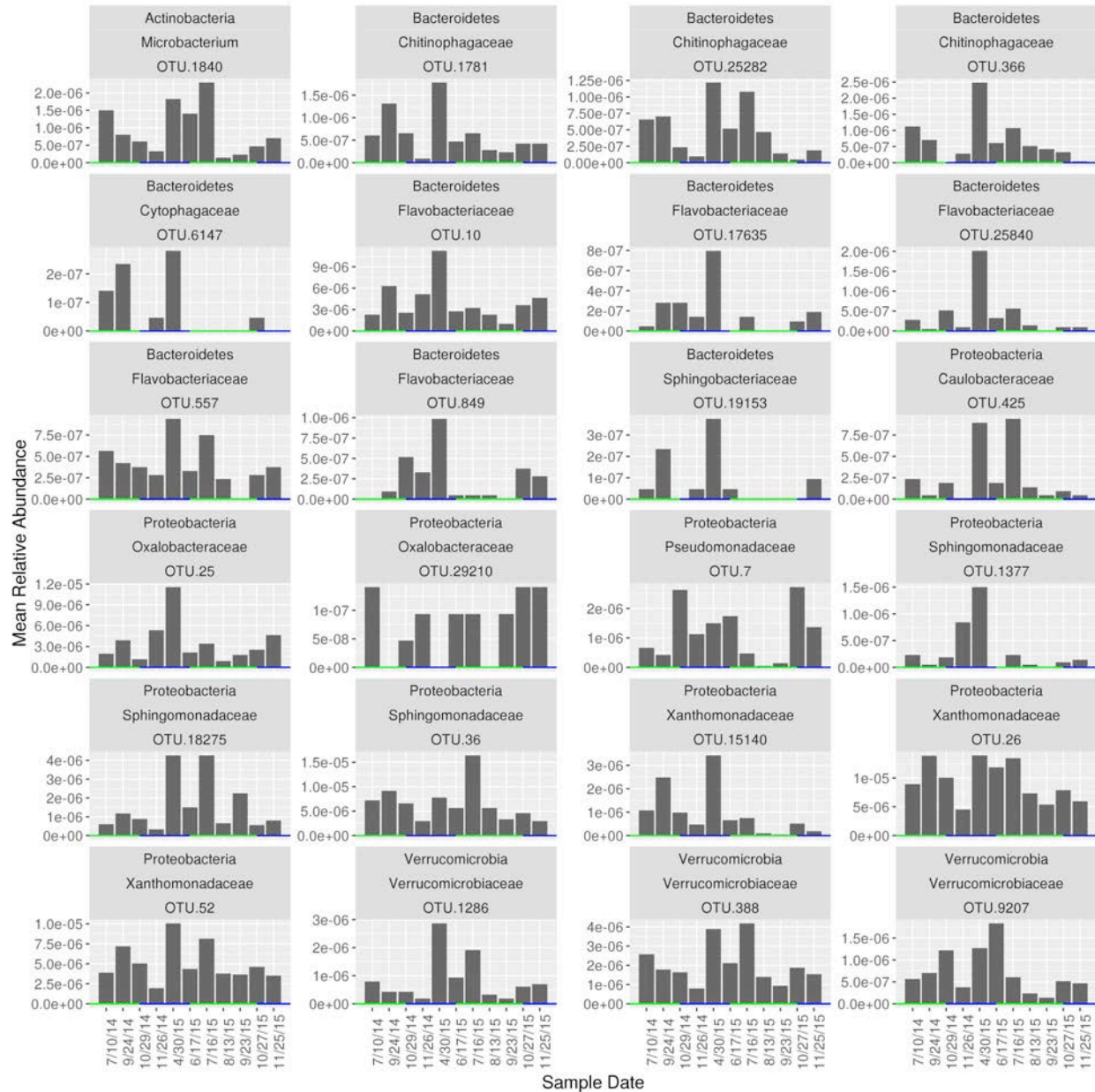




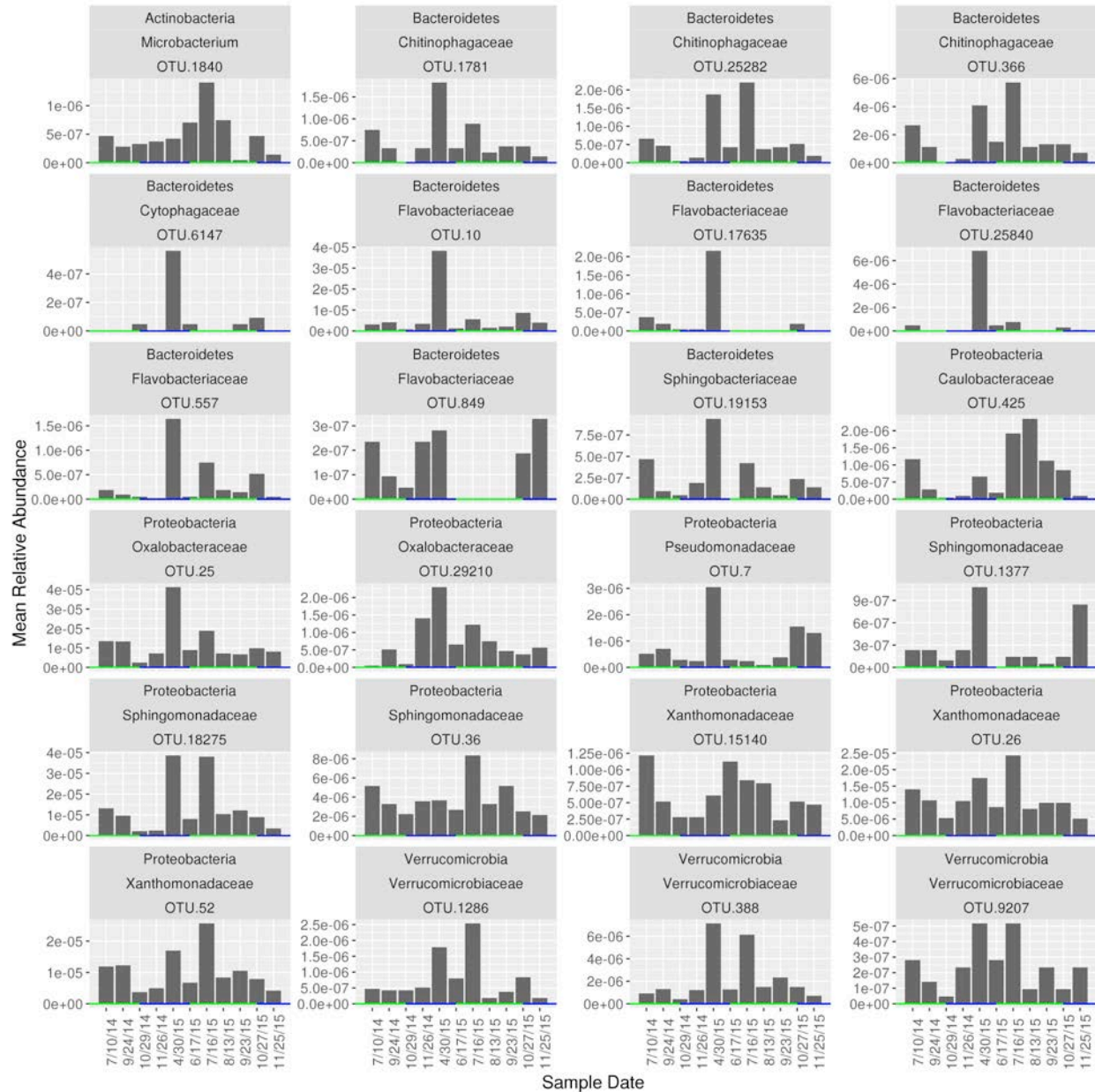
**Supplemental Figure 2.17.** Mean relative abundance over sampling time points in the NTR treatment of *Proteobacteria* OTUs identified as enriched in either April or November, as compared to August. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.



**Supplemental Figure 2.18.** Mean relative abundance over sampling time points in the NTR treatment of *Actinobacteria* OTUs identified as enriched in either April or November, as compared to August. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.

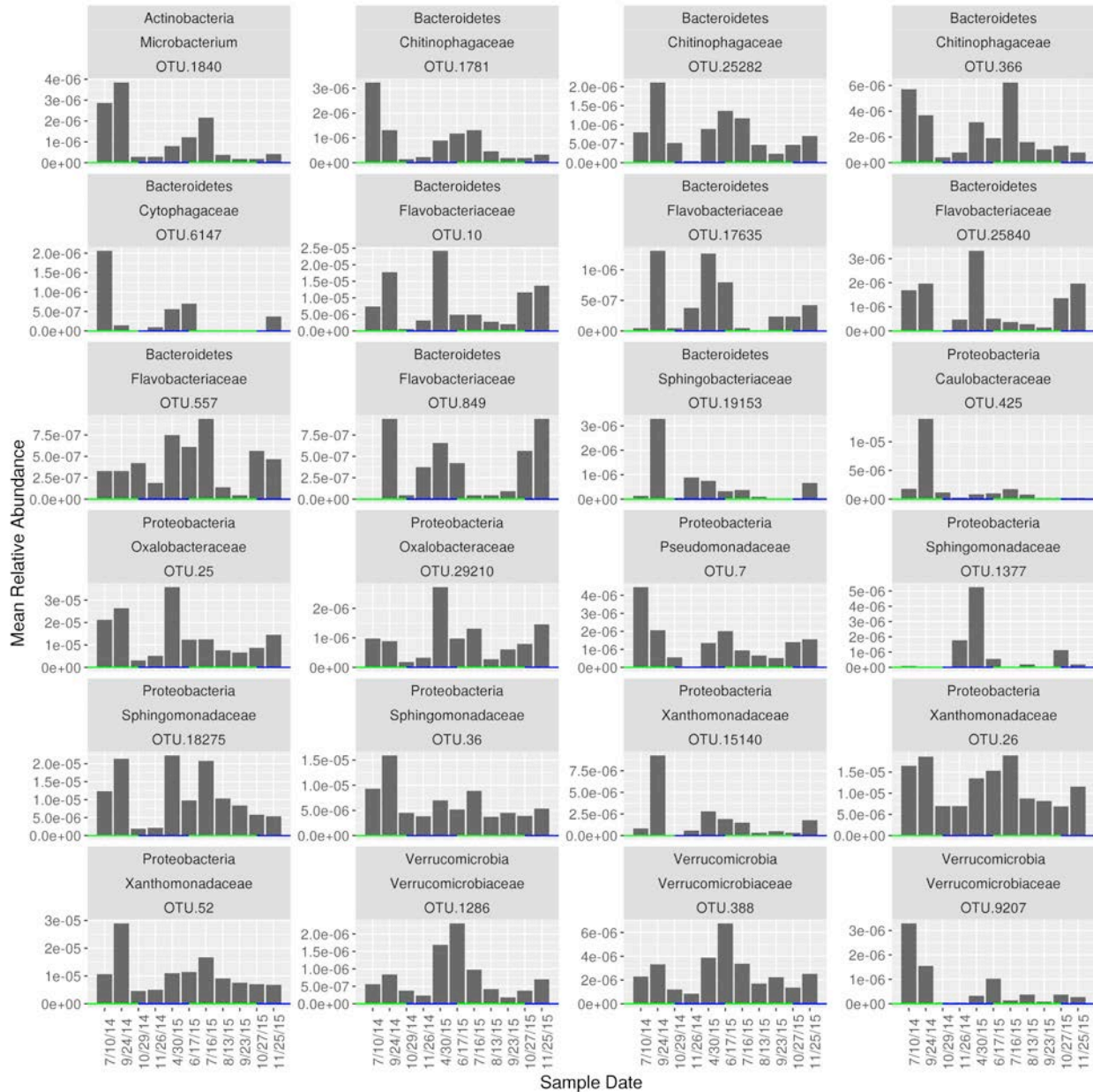


**Supplemental Figure 2.19.** Mean relative abundance over sampling time points in the NTH treatment of OTUs identified as enriched in either April or November NTR treatment, as compared to August NTR treatment. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.



**Supplemental Figure 2.20.** Mean relative abundance over sampling time points in the PTH treatment of OTUs identified as enriched in either April or November NTR treatment, as compared to August NTR treatment. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.





**Supplemental Figure 2.21.** Mean relative abundance over sampling time points in the PTH treatment of OTUs identified as enriched in either April or November NTR treatment, as compared to August NTR treatment. Colored bars at the bottom of the plot indicate growing (green color) and non-growing (blue color) seasons.

### **3 Chapter 3 : Fungal Community Composition Differs due to Land Management, Season, and Their Interactions**

#### **3.1 Abstract**

Fungal communities in the soil are highly diverse and essential to ecosystem processes such as carbon cycling. Land management and temporal variation impact these communities but the specific taxa affected are not known, which limits our understanding of fungal community dynamics and niches in the soils. Using high-throughput sequencing of the ITS region, we characterized the fungal community at a 42 year-old field site testing both tillage and biomass management. We took samples monthly in the growing seasons over two years to identify persistent community differences and differences that changed seasonally. The fungal community composition varied significantly due to season, tillage, and biomass management. We identified OTUs whose abundance varied across tillage or biomass management, with 61% of OTUs affected by land management showing enrichment in the no-till, biomass-returned treatment. We also identified OTUs that vary seasonally, and hypothesize that these OTUs may drive seasonal community changes in the no-till returned treatment due to differences in response to biomass management over time. The ability to identify specific OTUs affected by land management, time, and their interaction is a powerful tool for greater understanding of fungal niches in the soil ecosystem and the greater role of the fungal community in soil carbon cycling.

### 3.2 Introduction

Fungal communities in soil contribute to many ecosystem processes, including carbon cycling. Up to 80% of soil carbon cycling is mediated by the microbial community, including fungi (Treseder et al., 2012). The ability of fungi to produce extracellular enzymes that degrade complex plant matter containing lignin, hemicellulose, and cellulose has implicated them as essential degraders of complex and 'recalcitrant' carbon in the soil (IPCC, 2014). However, the understanding of fungal diversity in soil has been limited, as mycology has historically relied on cultivation-based or visual techniques. Around 100,000 fungal species have been identified, but diversity estimates suggest that there may be 0.8 – 1.5 million fungal species worldwide (Tedersoo et al., 2014).

General methods for analyzing fungal community composition include marker-based strategies, such as phospholipid fatty acid (PLFA) analysis, Automated Ribosomal Intergenic Spacer Analysis (ARISA), terminal restriction fragment length polymorphism (TRFLP), denaturing gradient gel electrophoresis (DGGE), or Sanger sequencing of clone libraries of small subunit (SSU) rRNA genes (Birgander et al., 2014; Kennedy et al., 2006; Schadt et al., 2003). PLFA analysis is one of the most popular for analyzing fungal (and microbial) communities. As phospholipids are thought to rapidly degrade upon organism death, the method provides a way to look at active populations and can be coupled with stable isotope probing to link function with identity (Frostegård and Bååth, 1996). However, a drawback of PLFA, as well as other fingerprinting methods, is low resolution and lack of specificity of phylogenetic groups

identified. Many studies link specific PLFA markers to discrete microbial groups (ex. saprotrophic fungi, gram positive bacteria, etc), but these connections are inferred from pure-culture studies and not always corroborated in different studies (Frostegård et al., 2011). Thus, PLFA data (and other fingerprinting data) can indicate a difference in the microbial community between two samples, but the specific organisms or groups of organisms that differ can be difficult to identify at high resolution. Fingerprinting techniques are also relatively low-throughput.

The advent of high-throughput sequencing (ie. Illumina sequencing) shows promise for higher resolution and higher-throughput studies of fungal communities. This technique relies on amplification and sequencing of a marker gene (ie. the internal transcribed spacer or ITS region). Sequences can be compared to marker databases to infer taxonomic annotation, and may also be studied in a taxonomic-independent manner through clustering into operational taxonomic unit (OTU) groups. High-throughput sequencing using the ITS region allows identification of taxa to the genus and potentially even the species level (Bates et al., 2013; Schoch et al., 2012). Additionally, with barcoding technology, hundreds samples can be sequenced on one sequencing run (Kozich et al., 2013).

Land management and temporal variation can both affect fungal communities in soil. Studies looking at land management effects on fungal communities show differences in community composition due to tillage and biomass management. (Carbonetto et al., 2014; Drijber et al., 2000; Frey et al., 1999; Jansa et al., 2002, 2003; Mbuthia et al., 2015; Miura et al., 2015; Neely et al., 1991; Sjöle et al., 2015; Spedding et al., 2004).

For example, arbuscular mycorrhizal fungi (AMF) biomarkers (Drijber et al., 2000; Jansa et al., 2002) and saprotrophic fungi biomarkers (Mbuthia et al., 2015; Miura et al., 2015) were found to be reduced in tilled as compared to no-till soils. The length of fungal hyphae and fungal biomass is higher in no-till vs till conditions (Frey et al., 1999), and fungal biomass is higher in biomass-retained conditions (Spedding et al., 2004).

Fungal communities also vary seasonally, although previous studies found that abiotic variables (such as pH, nitrogen, moisture, temperature, and land management) have greater impact on fungal community than does season (Birgander et al., 2014; Kennedy et al., 2006; Schadt et al., 2003). Fungal communities were found to shift in composition from the summer to the fall in grassland communities (Kennedy et al., 2006). Additionally, at least one study has examined seasonal dynamics of fungal communities over multiple land management practices. This study, conducted at a long-term tillage and biomass management field site during one growing season, found no interaction between date sampled and land management, but this may be due to their sampling times (May, June, July, and September) (Spedding et al., 2004).

Changes in fungal community composition can produce changes in carbon cycling (Treseder et al., 2012). Hence, if we are to understand the role of fungal communities in soil processes, it is important to explore the degree to which land management relates to variance in fungal community composition and how these responses vary seasonally. Incorporation of information about microbial communities into carbon cycling models can aid in model accuracy and prediction (Wieder et al., 2013). Therefore, understanding community changes due to land management helps model development

and greater comprehension of terrestrial carbon cycling.

Furthermore, little is known about fungal habitat preference or seasonal dynamics at a high taxonomic resolution. Thus, OTU-level assessment of fungal distribution among differing land managements and seasons allows for identification of specific groups of fungal OTUs that are enriched in one condition compared to another, and whether that enrichment has a phylogenetic basis. Using a replicated field site that tests effects of both tillage and biomass management, we sampled and characterized fungal communities using Illumina high-throughput sequencing of the ITS region over a period of two years to identify factors contributing to variation in soil fungal communities. To our knowledge, our study is the first to look at seasonal effects on fungal soil communities, and the first to explore seasonal differences across land managements using Illumina sequencing methodology. With this technique, we also identified specific OTUs that are influenced by each factor and investigated causes of temporal variation, including temperature and moisture.

### **3.3 Methods**

#### ***3.3.1 Soil Sampling***

We sampled soils from a long-term continuous corn tillage experiment, established in 1973, at the Miner Agricultural Research Institute in Chazy, NY (Clinton County, 44°53.13'N, 73°28.40'W. Experimental plots at the site all contain the same soil type – Raynham silt loam – and are part of a 2 x 2 factorial design that tests both tillage and maize biomass management. Thus, there are four effective treatments: tillage

(moldboard plowed and disked) with biomass returned (PTR), tillage with biomass harvested (PTH), no-till with biomass returned (NTR), and no-till with biomass harvested (NTH). Within the biomass returned treatment, grain is harvested but all other plant biomass is returned to the soil. The total plant biomass returned to the soil is around 8.4 – 8.5 Mg ha<sup>-1</sup> (Moebius-Clune et al., 2008). In the harvested treatment, plant biomass is removed for silage by cutting stalks adjacent to the soil surface, with only roots remaining. Harvest occurs in mid-October to early November and tilled plots are tilled by mid-November. In the harvested treatment, plant biomass is removed for silage by cutting stalks adjacent to the soil surface, with only roots remaining. Plots (6 x 15.2 m) are arranged in a randomized complete block, with four replicates for each treatment. Maize is fertilized according to standard agronomic practices for the region (26 kg Ha<sup>-1</sup> per year fertilizer N). Harvest occurs in mid-October to early November and tilled plots are tilled by mid-November.

We took soil samples from all replicate plots on eleven time points from July 2014 – November 2015 : 7/10/2014, 9/24/2014, 10/29/2014, 11/26/2014, 4/30/2015, 6/17/2015, 7/16/2015, 8/13/2015, 9/23/2015, 10/27/2015, 11/25/2015. For each plot, we collected 10 cores (2.5 cm diameter, 5 cm depth) along a transect that spanned the complete length of the plot. We also collected soil moisture and temperature data, using moisture and temperature probes, for three points within each plot (beginning, middle, and end of plot), and we averaged across the three points when analyzing data. Samples were transported on ice and cores for individual plots were homogenized and sieved to 2 mm. Once sieved, samples were stored at -80°C.

Additional soil analyses, including pH, soil C (in units of %), and soil N (in units of %) were done for one time point (9/24/2014). We measured pH using standard 1:1 soil-water method, and total soil C and N were determined on oven dried and ground samples via LECO Treu Mac CN-2000\* elemental analyzer (LECO Instruments, Lansing, MI) as previously described (Berthrong et al., 2013)

### **3.3.2 DNA Extraction**

We extracted DNA from two subsamples of 0.25 g of soil for each plot at each time point sampled using a modified Griffith's phenol-chloroform DNA extraction method (Griffiths et al., 2000; Pepe-Ranney et al., 2015). Duplicate extracts for each plot-time point were combined. Combined extracts were quantified using a PicoGreen assay (Invitrogen, Carlsbad, CA, USA).

Internal transcribed spacer 1 (ITS1) amplicons were generated from DNA by PCR using primer set nBITSf/58A2r (Bokulich and Mills, 2013; Martin and Rygielwicz, 2005). Primers were barcoded using the dual indexing scheme of Kozich et al. (Kozich et al., 2013). Each 25  $\mu$ L reaction contained 1x Q5 High Fidelity PCR Master Mix (New England Biolabs), 0.3  $\mu$ M of each primer, 1.25  $\mu$ L BSA, and 0.625  $\mu$ L of Picogreen reagent (for detection of successful amplification via fluorescence on qPCR machine). Reactions contained a normalized 5 ng of template, and each sample was amplified in triplicate. Thermal cycling occurred with an initial denaturation step of 30 seconds at 98°C, followed by 30 cycles of amplification (5s at 98°C, 30s at 50°C, 10s at 72°C), and a final extension step of 2 min at 72°C. Triplicate amplicons were pooled. Purification



and normalization was done using the SequalPrep normalization kit (Invitrogen), using manufacturer's protocol. Samples were concentrated down to 5 ng/uL, via vacuum centrifugation, and were sent to the Cornell Core Facility in Ithaca, NY to be run on an Illumina MiSeq Machine, using the V3 chemistry with 2 x 300 bp read length.

*In silico* testing of primers, using Primer Prospector (Walters et al., 2011), was done against the UNITE database (release its\_12\_11) (Kõljalg et al., 2013). Our forward primer hit between 30 – 77% of fungal sequences in each phylum represented in the database, while the reverse primer hit 49 – 100% (Supplementary Figure. 3.1). These primers have better *in silico* performance, than any other ITS1 or ITS2 primers described in the literature (Supplemental Table 1).

### **3.3.3 Post-Sequencing Analysis**

#### *Quality Control*

All code used for data processing and analysis can be found on Github ([https://github.com/chvtk/Chazy\\_Bulk\\_ITS](https://github.com/chvtk/Chazy_Bulk_ITS)).

Raw Illumina sequencing reads were merged with PEAR, using program defaults, and then demultiplexed (Zhang et al., 2014). Illumina sequences were then screened by maximum expected errors, with reads exceeding a maximum expected error threshold of 1.0 being discarded. Sequences with homopolymers greater than 8 bp were removed (Kozich et al., 2013).

#### *OTU Clustering and Taxonomic Assignment*

The dataset underwent OTU clustering using UParse (Edgar, 2013). Cluster

seeds were identified with a non-redundant set of reads, using USearch version 7.0.1090, with a sequence identity threshold for a new OTU centroid set at 97%. With USearch/UParse, potential chimeras were identified during OTU centroid selection and could not become cluster centroids, eliminating chimeras from the read pool. All quality controlled reads of the combined dataset were then mapped to cluster centroids at an identity threshold of 97%, again using USearch. 67.6% of quality controlled reads were mapped to centroids. Unmapped reads did not count towards sample counts and were removed from downstream analyses.

Reads were taxonomically annotated using the UClust taxonomic annotation framework in the QIIME software package with cluster seeds from the UNITE ITS database using 97% sequence identity OTUs as reference (version 7, release date 2016-01-31) (Caporaso et al., 2010; Edgar, 2010; Kõljalg et al., 2013). Reads annotated as “Chloroplast”, “Bacteria”, “Archaea”, “Unassigned” or “mitochondria” were removed from the dataset.

### *Bioinformatic Analyses*

Prior to statistical analyses, samples were subset to a range of 2,500 – 10,000 sequences due to greater than 4-fold differences in sequencing depth among samples (Weiss et al., 2015). In order to determine if sequencing depth impacted the interpretation of results, we uniformly rarefied samples to 2,500 sequences per sample and found similar ordination and PERMANOVA outcomes as compared to the non-rarefied and range-subset approach. The final dataset contained 1,012,878 sequences, with a mean of 5,755 and range of 2,500-10,000 sequences per sample, and 2,247

identified OTUs.

R version 3.1.2 was used for all analyses, utilizing the phyloseq, vegan, dplyr, DESeq2, and ggplot2 libraries (Love et al., 2014; McMurdie and Holmes, 2013; R Core Team, 2014; Wickham and Chang, 2015; Wickham et al., 2015)

### *Climate Data Analysis*

Daily precipitation and ambient air temperature data was obtained from NOAA, using the Plattsburgh International Airport weather station (44.6597° N, 73.4672° W).

### *NMDS Ordination*

Bray-Curtis was used to calculate a phylogenetic distance matrix for samples (Bray and Curtis, 1957). The distance matrix was visualized using Nonmetric Multidimensional Scaling (NMDS). Adonis statistics (Permutational Multivariate Analysis of Variance) were used to partition ordination variance attributed to various treatment conditions. Since the experiment consisted of a randomized complete block design we included replicate block as a strata term in all models.

### *Differential Expression Statistical Framework*

The RNA-Seq differential expression statistical framework (DESeq2) was used to identify OTUs that were enriched in different treatment conditions (Anders and Huber, 2010; McMurdie and Holmes, 2014). “Differential abundance” refers to different sample means for an individual OTU in contrasting treatments (eg. No-till vs till). DESeq2 was used to calculate the moderated log<sub>2</sub>-fold change proportion mean ratios and corresponding standard errors for variables of temperature, moisture, month (specifically August vs November), tillage, and biomass management (Anders and

Huber, 2010). P-values were corrected for multiple comparison with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). OTUs from the dataset were defined as incorporators if they had an adjusted p-value less than the false discovery rate of 0.10.

As a form of independent filtering, a sparsity filter was applied to the dataset (Bourgon et al., 2010). A sparsity filter removes low frequency and statistically uninformative OTUs that are not found in equal or greater fraction of samples as the sparsity threshold. To find greatest number of differentially enriched OTUs, we tested sparsity thresholds from 0.05 – 0.95 in steps of 0.05 for each  $\log_2$ -fold change calculation (Pepe-Ranney et al., 2016). A sparsity threshold of 0.05 was chosen, resulting in a dataset of 737 OTUs.

### **3.4 Results**

#### ***3.4.1 Soil and Environmental Factors***

Soil chemistry differed between field treatments, but environmental factors did not. The main effect of tillage and biomass removal was significantly reduced soil C and soil N (Table 3.1, soil C, tillage - F-value = 77.2,  $p < 0.001$ ; soil C, biomass management - F-value = 13.7,  $p = 0.005$ ; soil N, tillage - F-value = 127.9,  $p < 0.001$ ; soil N, biomass management - F-value = 7.7,  $p = 0.02$ ). The main effect of tillage, but not biomass management significantly decreased soil C:N (Table 3.1, C:N, tillage- F-value = 14.7,  $p = 0.004$ ) . We observed a significant interaction of tillage with biomass management for soil C but not for soil N or C:N (soil C - F-value = 6.4,  $p = 0.03$ ). NTR plots have

significantly higher soil C than NTH and till plots, and NTH plots have higher soil C than till plots, but till plots that varied in biomass conditions do not significantly differ in soil C (Table 3.1, Pairwise and paired t-test,  $p = 0.03$ ). Soil N is significantly higher in both tilled treatments as compared to no-till treatments but, within tillage, treatments did not differ significantly by biomass management (Table 3.1, Pairwise and paired t-test,  $p = 0.3$ ). Soil C:N ratios range from 11.2 -17.3, but are only significantly different for NTH and PTH treatments (Table 3.1, Pairwise and paired t-test,  $p = 0.05$ ). No significant differences exist between soil temperature, moisture, or pH between treatment plots (Supplementary Figure 3.2, Table 3.1).

**Table 3.1.** Soil characteristics of the long term tillage experiment in Chazy, NY. All measurements, except moisture, were taken from soil in September 2014. Moisture was averaged over the 11 sampling timepoints from July 2014 – November 2015. Differing superscript letters in each column indicate significant differences between treatments

Treatment	Tillage	Biomass Retention	%C	%N	C:N	pH	% Moisture
<b>NTR</b>	No	Yes	2.77 ± 0.48 <sup>a</sup>	0.23 ± 0.03 <sup>a</sup>	12.20 ± 0.68 <sup>ab</sup>	6.89 ± 0.68 <sup>a</sup>	17.1 ± 3.07 <sup>a</sup>
<b>NTH</b>	No	No	2.13 ± 0.39 <sup>b</sup>	0.18 ± 0.04 <sup>a</sup>	11.58 ± 0.32 <sup>a</sup>	6.89 ± 0.93 <sup>a</sup>	16.5 ± 2.47 <sup>a</sup>
<b>PTR</b>	Yes	Yes	1.62 ± 0.40 <sup>c</sup>	0.11 ± 0.01 <sup>b</sup>	14.36 ± 2.02 <sup>ab</sup>	7.66 ± 0.07 <sup>a</sup>	17.4 ± 8.19 <sup>a</sup>
<b>PTH</b>	Yes	No	1.50 ± 0.26 <sup>c</sup>	0.10 ± 0.02 <sup>b</sup>	14.36 ± 1.12 <sup>b</sup>	7.74 ± 0.05 <sup>a</sup>	16.5 ± 6.59 <sup>a</sup>

Temperature and precipitation varied over the sampling time period

(Supplemental Figures 3.2a, 3.2b). No significant inter-annual variation in precipitation

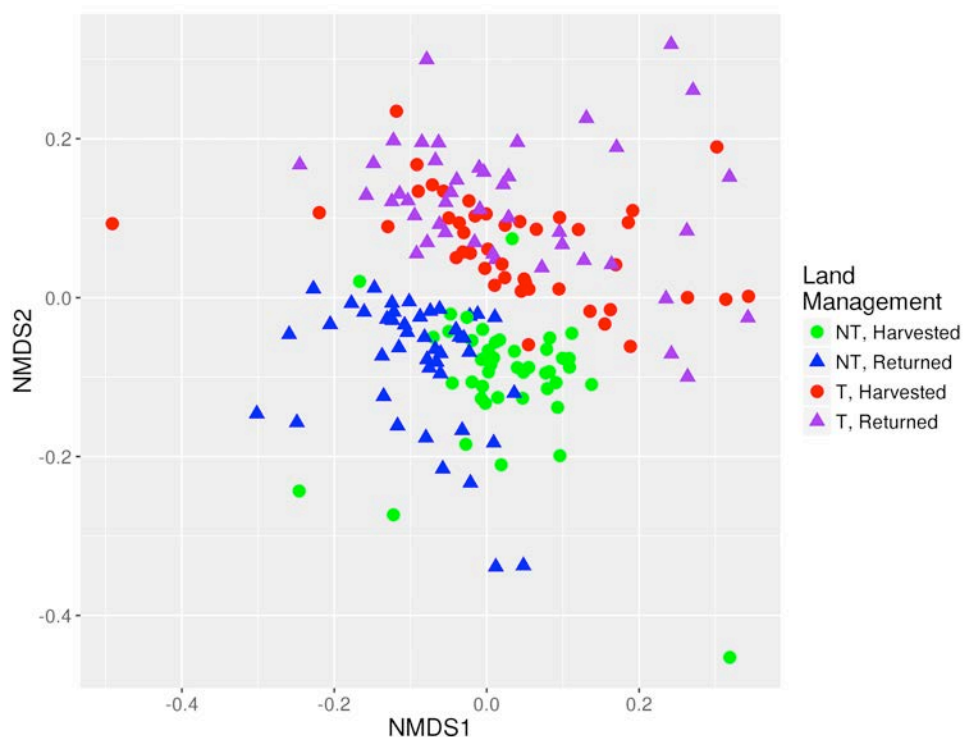
or air temperature occurred over the two years of sampling (full years 2014 and 2015 were compared, t-test, precipitation:  $p = 0.8$ , air temperature:  $p = 0.3$ ). However, precipitation differed when comparing sampling date (Supplemental Figure 3.2b, ANOVA,  $p < 0.001$ , F-value = 2.7). This seemed to be driven by high precipitation in June 2015 (Tukey's HSD). Precipitation was highest during the growing season (May – August) and was lower outside of the growing season (September – April) (Supplemental Figure 3.2b).

### ***3.4.2 Fungal Community Composition Varies with Land Management and Time***

We identified 2,247 fungal OTUs from 1,012,878 sequences. Treatments did not differ significantly in OTU number, with 1,343 OTUs detected in NTR, 1,363 OTUs in NTH, 1,178 OTUs in PTR, 1,314 OTUs in PTH. After applying a sparsity threshold of 0.05 for bioinformatics analyses, 737 OTUs remained in the dataset. Chao1 diversity scores were significantly higher in no-till treatments than till treatments (Supplemental Table 3.2; ANOVA,  $p < 0.001$ , F-value = 13.0), but did not significantly differ by biomass management (ANOVA,  $p = 0.26$ , F-value = 1.3). Evenness, as measured by Pielou's Evenness also differed by tillage, but not by biomass management (Supplemental Table 3.2; tillage : ANOVA,  $p = 0.002$ , F-value = 9.6; biomass: ANOVA,  $p = 0.2$ , F-value = 1.5). Both differences in evenness and richness were driven by the PTR treatment (Tukey's HSD; Supplemental Table 3.2).

NMDS ordination of Bray-Curtis distance dissimilarities between ITS sequences indicates that both land management and temporal variation drove differences in fungal community composition (Figure 3.1, Figure 3.2). Land management contributed 16.7 %

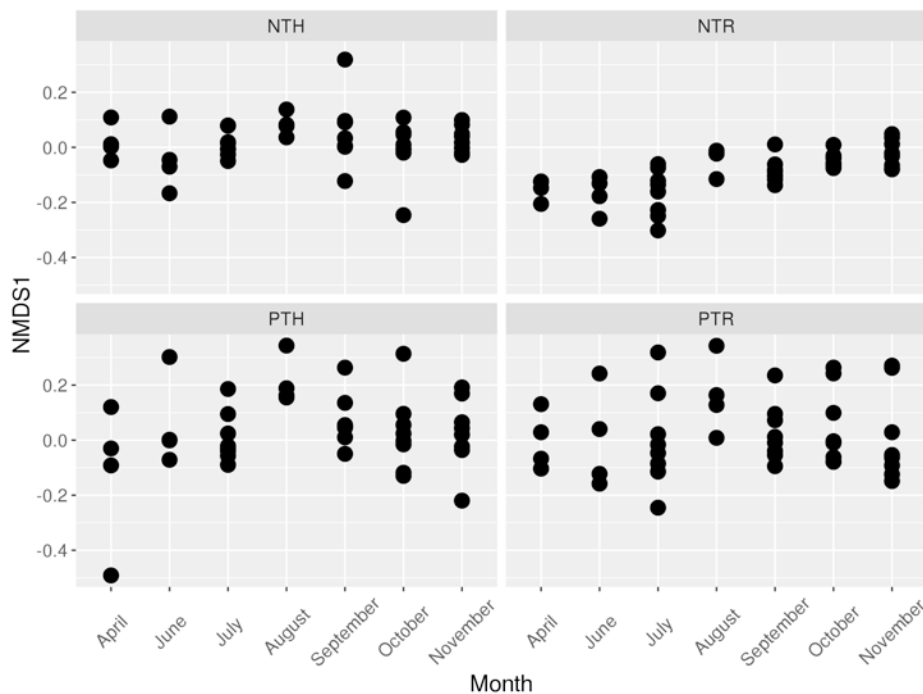
of variation in community composition (PERMANOVA,  $p$ -value = 0.001, F-statistic = 13.1), and temporal variation (time-point sampled) contributed 13.9% (PERMANOVA,  $p$ -value = 0.001, F-statistic = 3.3). The month sampled (irrelevant of year) explained 8.9% of community variance (PERMANOVA,  $p$ -value = 0.001, F-statistic = 3.3). When the effect of land management was assessed across time, 10.5% of community variation was due to tillage (PERMANOVA,  $p$ -value = 0.001, F-statistic = 24.8) and 4.3% of variation was due to biomass management (PERMANOVA,  $p$ -value = 0.001, F-statistic = 10.1).



**Figure 3.1.** NMDS ordination of Bray-Curtis distances for fungal communities at each sampling time-land management combination. Colors represent land-management and shapes represent biomass status (circles – harvested, triangles – returned)

Variation in the fungal community was also attributed to interactions between variables. The interaction term that explained the most variation is between land

management and time (PERMANOVA,  $p$ -value = 0.005,  $R^2$  = 0.136, F-statistic = 1.1). In decomposing this interaction, significant variance was explained by the interaction of tillage and time (PERMANOVA,  $p$ -value = 0.005,  $R^2$  = 0.06, F-value = 1.4) but not by the interaction of biomass management and time (PERMANOVA,  $p$ -value = 0.12,  $R^2$  = 0.04, F-statistic = 1.13). There was also a significant interaction between tillage and biomass management (PERMANOVA,  $p$  = 0.001,  $R^2$  = 0.02, F-statistic = 4.5).



**Figure 3.2.** NMDS1 values vs month sampled for fungal communities at each sampling time-treatment combination. The figure is faceted into the four agricultural treatments.

Examined by treatment, both the tilled and no-till treatments varied significantly with time (PERMANOVA, till:  $p$  = 0.001,  $R^2$  = 0.21, F-statistic = 2.1; no-till :  $p$  = 0.001,  $R^2$  = 0.22, F-statistic = 2.6). Biomass management explained greater variation within the no-till treatment (PERMANOVA,  $p$  = 0.001,  $R^2$  = 0.10, F-statistic = 11.4) than within the tilled treatment (PERMANOVA, till:  $p$  = 0.001,  $R^2$  = 0.04, F-statistic = 4.1). In

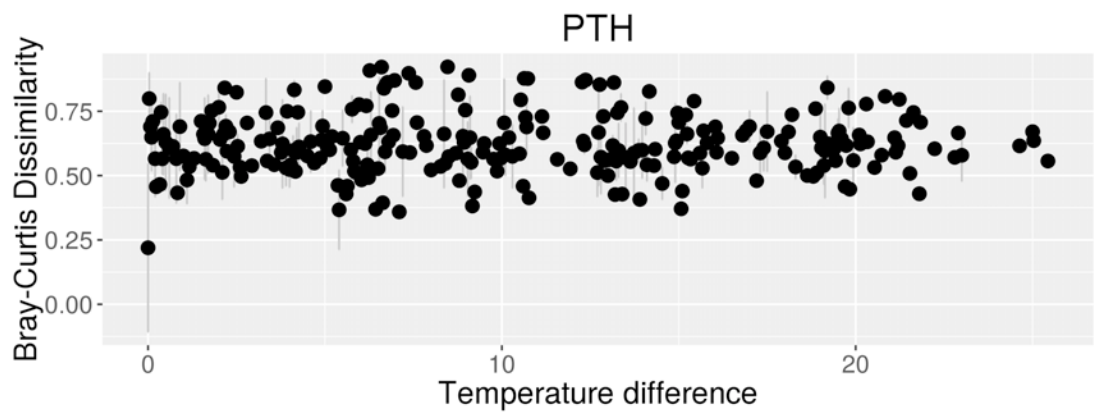
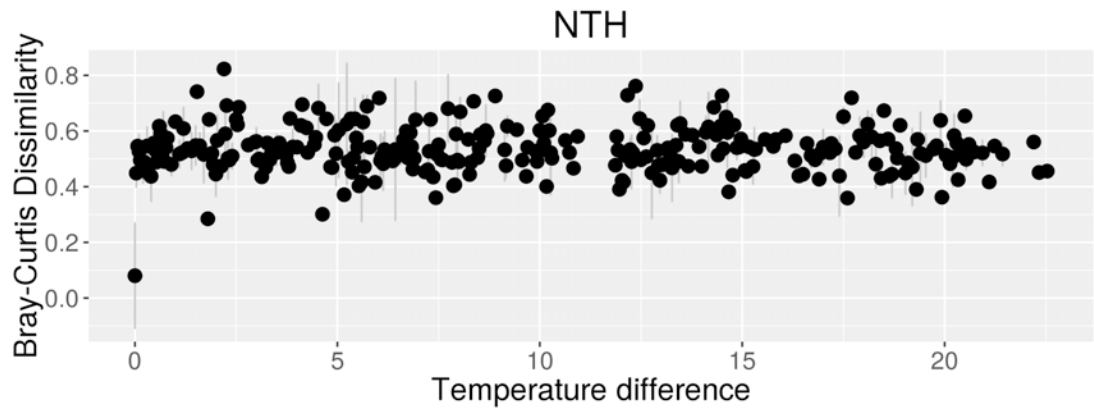
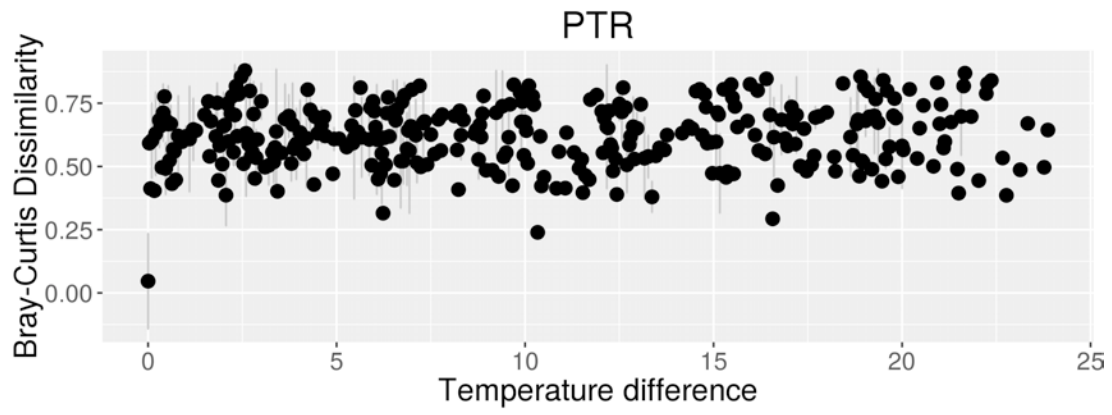
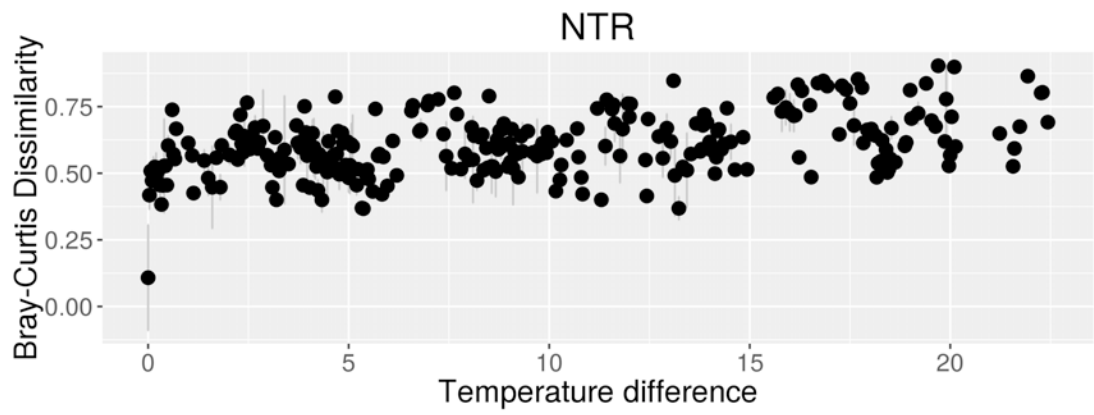


addition, there was a significant interaction between biomass management and time within the no-till treatment (PERMANOVA,  $p = 0.005$   $R^2 = 0.11$ , F-statistic = 1.3) but not within the tilled treatment (PERMANOVA,  $p = 0.99$   $R^2 = 0.06$ , F-statistic = 0.66).

Samples from plots with returned biomass and removed biomass showed variation with time (PERMANOVA, biomass returned:  $p = 0.001$ ,  $R^2 = 0.20$ , F-statistic = 2.4; biomass harvested:  $p = 0.001$ ,  $R^2 = 0.18$ , F-statistic = 2.0) and tillage (PERMANOVA, biomass returned :  $p = 0.001$ ,  $R^2 = 0.14$ , F-statistic = 16.6; biomass harvested :  $p = 0.001$ ,  $R^2 = 0.12$ , F-statistic = 12.7). There was also a significant interaction between tillage and time within the biomass returned treatment (PERMANOVA,  $p = 0.007$ ,  $R^2 = 0.10$ , F-statistic = 1.2), but not within the biomass harvested treatment (PERMANOVA,  $p = 0.19$ ,  $R^2 = 0.09$ , F-statistic = 0.94).

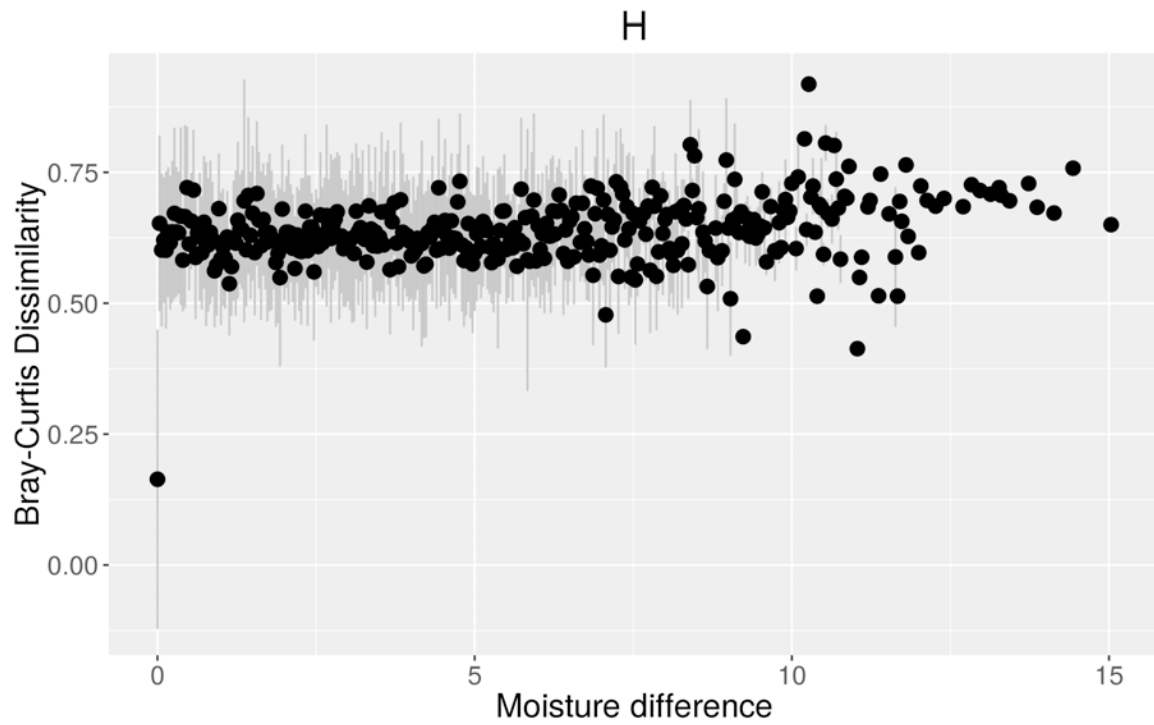
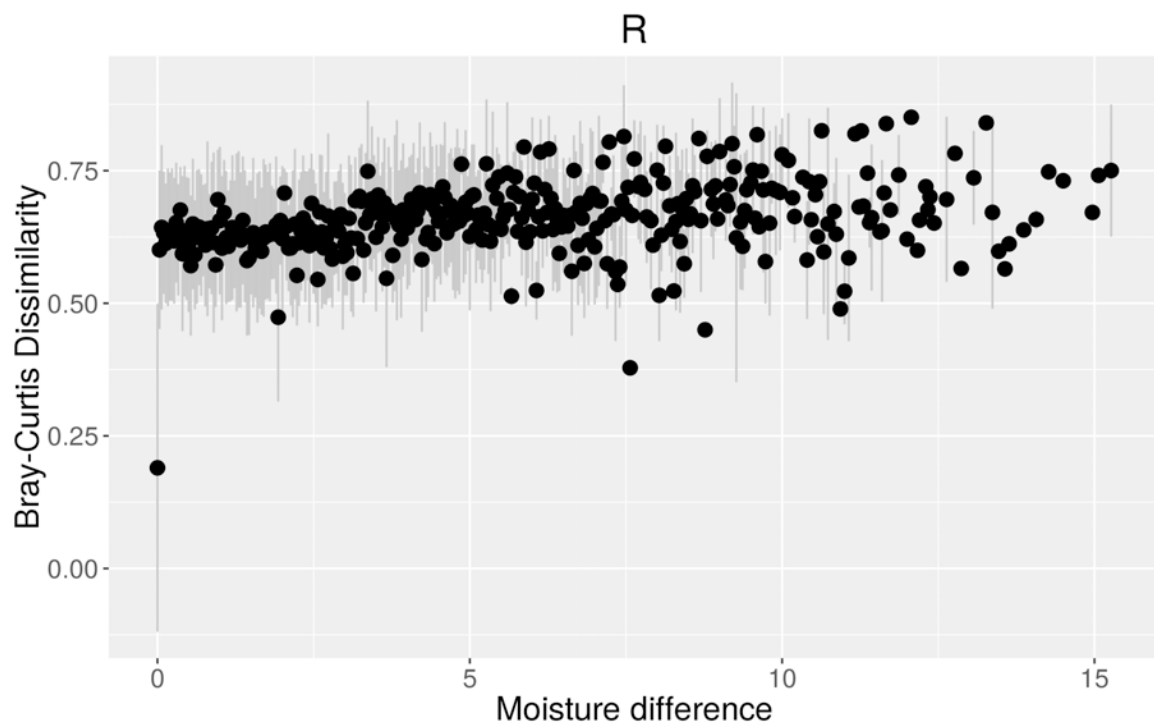
### ***3.4.3 Differences in Soil Temperature and Moisture Correlate with Differences in Fungal Community Composition***

We looked at soil temperature and moisture as proxy variables representing seasonal change within our dataset, and found that they correlated with differences in community composition among samples. Specifically, Bray-Curtis dissimilarity values for pairwise comparisons of samples were correlated to pairwise temperature differences (Mantel test,  $p = 0.0037$ ,  $R^2 = 0.07$ ). That is, increasing difference in temperature correlated with increasing phylogenetic distance in fungal sequences. Separating samples by land management indicated that NTR samples drove this correlation (Figure 3.3, NTR:  $p = 0.0001$ ,  $R^2 = 0.41$ ; other land managements were not significant).



**Figure 3.3.** Bray-Curtis dissimilarity pairwise distances vs. pairwise temperature distances for samples within the same land management. Community dissimilarity and temperature differences correlated significantly for the NTR treatment but not for other treatments (Mantel test,  $p = 0.0001$ ,  $R^2 = 0.41$ ).

We also examined the correlation between soil moisture and fungal community variation and found that it was also significant (Mantel,  $p = 0.002$ ,  $R^2 = 0.12$ ). Splitting samples by land management shows that returned-biomass samples have more variation correlated with soil moisture than harvested biomass samples (Figure 3.4, Mantel; returned samples:  $p = 0.005$ ,  $R^2 = 0.17$ ; harvested samples:  $p = 0.10$ ). Within returned biomass samples, those under till management had a slightly stronger correlation than those under no-till (Supplemental Figure 3.3, PTR :  $p = 0.02$ ,  $R^2 = 0.18$ ; NTR:  $p = 0.04$ ,  $R^2 = 0.14$ ).



**Figure 3.4.** Bray-Curtis dissimilarity pairwise distances vs. pairwise temperature distances for samples in returned biomass (top) or harvested biomass (bottom) treatment. The returned biomass samples have a significant correlation between the two variables ( $p = 0.004$ ,  $R^2 = 0.17$ ) while harvested biomass do not.

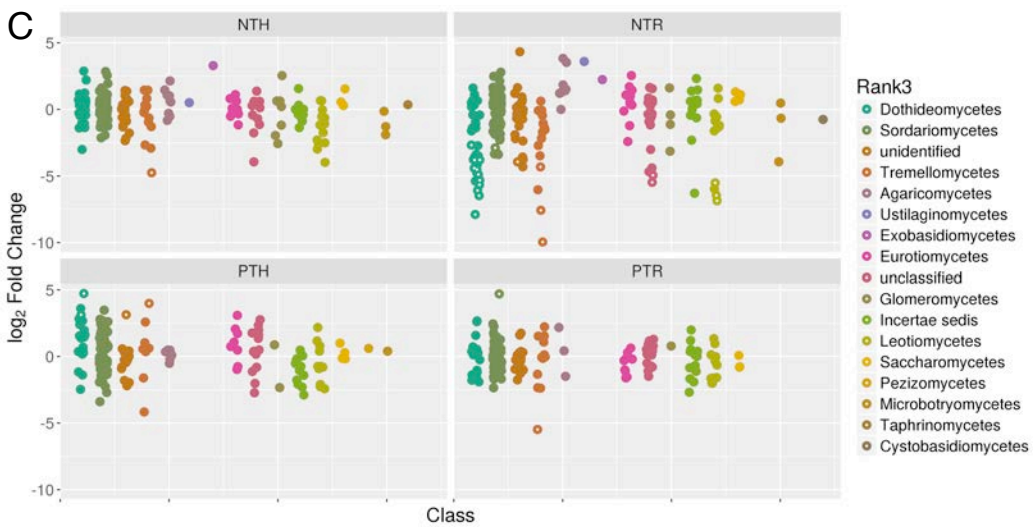
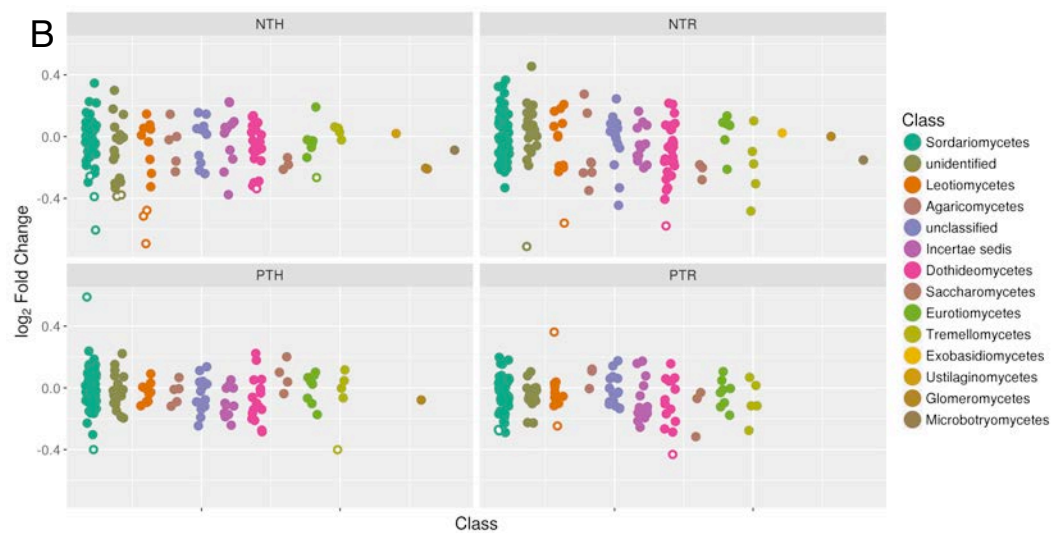
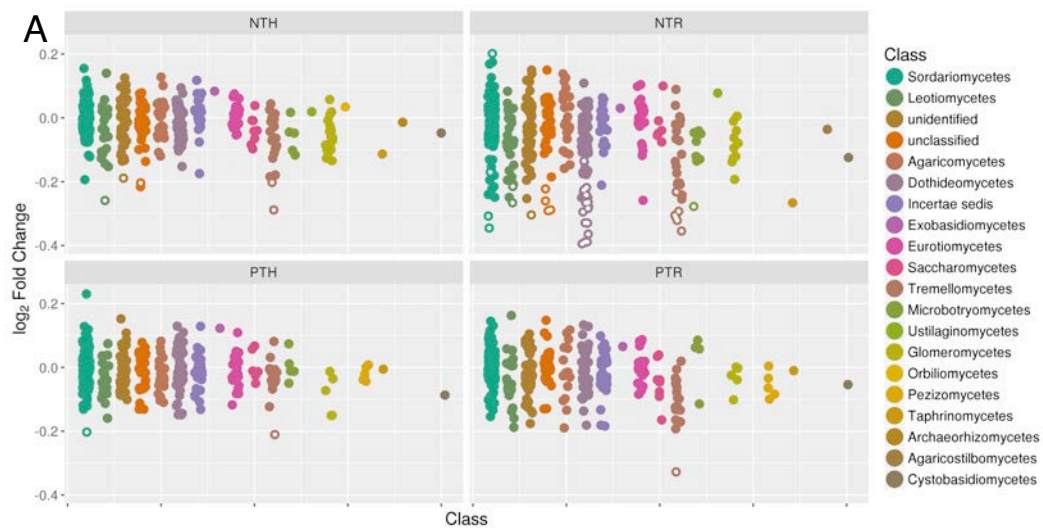
#### **3.4.4 OTUs from Classes *Dothideomycetes* and *Tremellomycetes* Vary with Temperature**

We calculated the differential abundance of fungal OTUs as a function of soil temperature in each treatment (Figure 3.5a). Coefficients obtained from this analysis indicate the change in OTU differential abundance in response to temperature. Of OTUs tested, 43 (7.6%) were found to have statistically significant coefficients, indicating a significant change in abundance in relation to temperature (adjusted  $P < 0.10$ , Wald test). Of these, 42 OTUs (34 from NTR, 5 from NTH, 2 from PTH, 1 from PTR) had negative correlations between abundance and temperature and 1 OTU (NTR, genus *Schizothecium*) had positive correlations between abundance and temperature. The mean positive  $\log_2$ -fold change correlation coefficient was 0.21, while the negative mean  $\log_2$ -fold change coefficient was  $-0.27 \pm 0.06$ . Most OTUs identified as decreasing in abundance with temperature belonged to the classes *Dothideomycetes* (15 OTUs, present in NTR; unidentified at genus level, orders: *Pleosporales*, *Capnodiales*, and unknown) and *Tremellomycetes* (10 OTUs, present in all treatments; genera *Dioszegia*, *Bullera*, *Hannaella*, *Cryptococcus*, *Mrakiella*, and unidentified). Other classes included *Leotiomyces* (3 OTUs, present in NTH and NTR; genera: *Hymenoscyphus* and unknown), *Microbotryomycetes* (1 OTU, NTR; genus *Sporobolomyces*), and

*Sordariomycetes* (4 OTUs, NTR and PTH; genera *Schizothecium*, *Microdochium*, and unknown). An additional seven OTUs were unidentified or unclassified at the class level. The abundance of three OTUs was significantly affected by temperature in both NTR and NTH treatments (OTU.1177 – genus *Dioszegia*, OTU.1851 – phylum *Ascomycetes*, OTU.692 – genus *Hymenoscyphus*, in NTR and NTH) while the relative abundance of one (OTU.549 – class *Tremellomycetes*) was significantly affected by temperature across all treatments.

### **3.4.5 OTUs from *Sordariomycetes* and *Leotiomyces* Vary with Moisture**

We also calculated the differential abundance of fungal OTUs as a function of soil moisture in each treatment (Figure 3.5b). Coefficients obtained from this analysis indicate the change in OTU differential abundance in response to moisture. Of OTUs tested, 21 (8%) were found to have statistically significant coefficients (adjusted  $P < 0.10$ , Wald test). Of these, 19 OTUs had negative correlations with moisture, while 2 had positive correlations. The mean positive  $\log_2$ -fold change coefficient was  $0.47 \pm 0.15$ , while the negative was  $-0.43 \pm 0.16$ . OTUs with negative correlation were annotated as members of *Sordariomycetes* (6 OTUs, from PTH, PTR, NTH; genera *Fusarium*, *Trichoderma*, and *Myrmecridium*), *Leotiomyces* (5 OTUs, from NTR, PTR, NTH; genera *Phialocephala*, *Hymenoscyphus*), *Dothideomycetes* (3 OTUs, from NTH, NTR, PTR; genus *Dreschlera*), *Eurotiomyces* (1 OTU, NTH; genus *Exophiala*), *Tremellomycetes* (1 OTU, PTH; genus unidentified). Three OTUs were unidentified or unclassified at the class level. The two OTUs with positive correlation were identified as



**Figure 3.5.** Log<sub>2</sub>-fold change of OTUs as a factor of (A) temperature, (B) moisture, or (C) enrichment in August or November. For (A) and (B) Log<sub>2</sub>-fold change values represent the change in OTU abundance with each degree in temperature or percent unit of moisture. OTUs with significant log<sub>2</sub>-fold change values are marked with a white dot. For (C) positive values indicate enrichment in August while negative values indicate enrichment in November. OTUs are faceted by land management, and ordered and colored by class.

*Leotiomyces* (PTR; genus *Neobulgaria*) and *Sordariomyces* (PTH; genus *Acremonium*).

The abundance of one OTU is significantly affected by moisture in both NTR and NTH treatments (OTU.197; *Helotiales* sp.) The relative abundance of five OTUs (OTU.227, OTU.692, OTU.549, OTU.26, and OTU.37716) are significantly impacted by both moisture and temperature conditions. Of these five OTUs, only OTU.227 (family *Lasiosphaeriaceae*) and OTU.37716 (class *Dothidiomycetes*) are annotated past the kingdom level.

### **3.4.6 OTUs Enriched in Fall also Respond to Temperature**

To explore effect of crop inputs across season, we compared OTU abundance when biomass input is greatest (November) to OTU abundance in the growing season when biomass input is small (August) across all four treatments. In November, crop biomass has been applied to the biomass returned treatments, whereas in August, plant biomass has been degrading for about 10 months and plant root exudation is the source of carbon into the soil microbial community. We identified 24 differentially enriched OTUs (adjusted  $P < 0.10$ , Wald test). The majority (21) of differentially enriched OTUs came from the NTR treatment and were enriched in November as compared to August



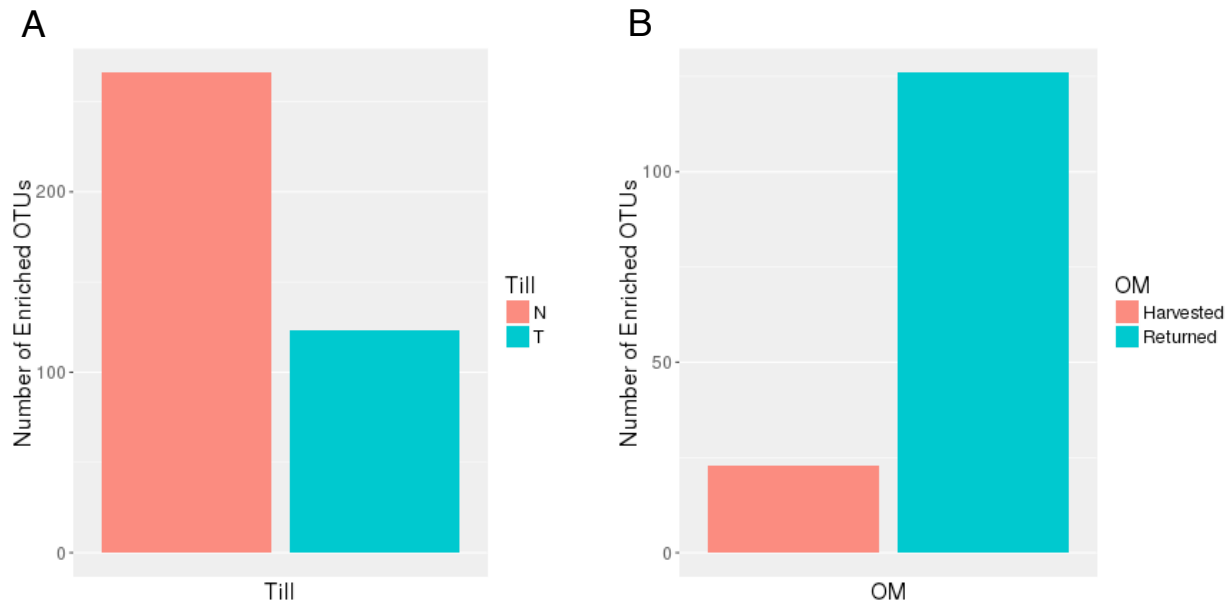
(Figure 3.5c). Fifteen of these OTUs overlapped with OTUs whose abundance correlated negatively to temperature in the NTR treatment (Figure 3.6). The overlap of OTUs enriched in these two conditions suggests enrichment of OTUs under biomass addition conditions. Thus, these fifteen OTUs may be driving the interaction between biomass, time, and tillage that we see reflected in changes in the community composition in the NTR treatment but not in the PTR treatment. These OTUs are annotated as belonging to genera *Dioszegia*, *Hymenoscyphus*, *Leptosphaeria*, *Mrakiella*, in addition to OTUs unable to be annotated at the genus level but annotated as members of the classes *Dothideomycetes* and *Tremellomycetes*. Additionally, two OTUs overlapped with OTUs whose abundance correlated to moisture difference. One of these OTUs can only be annotated as a member of Fungi, while the other is annotated to the class *Dothideomycetes*.

#### **3.4.7 Enrichment of OTUs under Tillage Condition Differs by Taxonomic Group but Favors No-till Condition**

We also explored the effect of treatment on OTU abundance by testing the differential enrichment of OTUs in either the no-till or till treatment condition. With this analysis, coefficients represent the change in abundance of an OTU in no-till vs till treatment. Positive coefficients represent enrichment in till conditions while negative coefficients represent enrichment in no-till conditions. We found 389 OTUs (53% of OTUs tested) with significant differential enrichment (adjusted  $P < 0.10$ , Wald test). Of these, 266 OTUs (68%) were enriched in the no-till treatment, while 123 (32%) were

enriched in the till treatment (Figure 3.6a). Differentially enriched OTUs came from several phyla including *Ascomycota* (58% of *Ascomycota* OTUs were differentially enriched and made up a total of 75% of all differentially enriched OTUs), *Basidiomycota* (29%, 8%), *Glomeromycota* (50%, 3%), *Rozellomycota* (83%, 1.2%), *Zygomycota* (46%, 4%). Enrichment differed at the phylum level (Supplemental Figure 3.5). For instance, 25 OTUs annotated as *Basidiomycetes* were significantly enriched in no-till conditions as compared to one OTU in till conditions (Supplementary Figure 3.6). However, OTUs annotated as *Ascomycetes* were more evenly split, with 193 OTUs enriched under no-till conditions and 97 OTUs enriched under tillage conditions (Supplemental Figure 3.7). Similarly, OTUs annotated as *Zygomycetes* were evenly split : 7 OTUs enriched under no-till and 7 OTUs enriched under till. *Glomeromycota* and *Rozellomycota* OTUs were more often significantly enriched under no-till conditions.

Many of these OTUs were unable to be assigned to genera. However, those that can came from 102 genera, with the most numerous being *Penicillium* (enriched in no-till), *Fusarium* (enriched in no-till), *Mortierella* (similar numbers of OTUs enriched in till and in no-till), *Acremonium* (enriched in till), *Myrmecridium* (enriched in no-till), and *Trichoderma* (enriched in no-till).



**Figure 3.6.** Number of differentially enriched OTUs in (A) tillage condition or (B) biomass management treatment.

### 3.4.8 More OTUs are Enriched in Returned Biomass Management as compared to Harvested Biomass Management

149 OTUs (20% of OTUs tested) differed in enrichment due to biomass management (adjusted  $p < 0.10$ , Wald test). Of those, 15% (23 OTUs) showed enrichment in biomass-harvested conditions, while 85% (126 OTUs) showed enrichment in biomass-returned conditions (Figure 3.6b). Most OTUs that differed significantly were found to have higher abundance in the returned biomass treatment (Supplemental Figure 3.8). Significantly enriched OTUs annotated as *Basidiomycetes* were enriched heavily in the returned treatment (17 OTUs) as compared to the harvested treatment (2 OTUs) (Supplemental Figure 3.9). *Ascomycetes* followed the same pattern (97 OTUs vs 19 OTUs, Supplemental Figure 3.10), while *Blastocladiomycota* and *Rozellomycota* are only differentially enriched in the returned

treatment (1 and 2 OTUs, respectively). *Zygomycota* OTUs were split between returned (2 OTUs) and harvested (3 OTUs) treatments. Again, most of these OTUs were unable to be assigned at the genus level. Those that can be assigned fall into 44 genera. The genera with most differentially enriched OTUs include *Cryptococcus* (enriched in biomass returned), *Trichoderma* (enriched in biomass returned), *Candida* (enriched in biomass returned), and *Penicillium* (enriched in biomass returned).

We find 343 OTUs whose abundance was significantly affected by both tillage and biomass management. Of these, 210 OTUs (61%) were significantly enriched in both no-till and returned biomass management, 83 (24%) in till and returned biomass management, 38 (11%) in no-till and harvested biomass management, and 12 (3.5%) in till and harvested biomass management.

### **3.5 Discussion**

#### ***3.5.1 Several Sources of Variation in Fungal Community Composition***

We identified 2,247 fungal OTUs and tracked changes in their relative abundance with respect to land management and time. Temporal variation accounted for more variation than tillage or biomass management. The month sampled accounted for a large portion of temporal variation but not all, suggesting that there is also some inter-annual variation in fungal communities. We also saw that fungal communities under different land managements differed over time, demonstrating interactions between land management and time.

### ***3.5.2 Temperature and Moisture as Proxies for Seasonal Community Change Due to Biomass Application***

We used temperature and moisture as proxies for testing seasonal variation in fungal communities. Temperature and moisture explained significant variation in fungal community composition in our study and in others (Dumbrell et al., 2011; Schadt et al., 2003; Tedersoo et al., 2014). For example, there is evidence of distinct AMF communities in summer and winter, attributed to changes in host-plant carbon and higher alpha diversity in the winter community due to increased competition with decreasing carbon sources (Dumbrell et al., 2011). Fungal biomass also increases in winter tundra soils and significant fungal community shifts have been correlated to changes in soil temperature, moisture, and carbon availability (Schadt et al., 2003).

The influence of temperature and moisture differed depending on land management. Specifically, a significant response to moisture was seen in the biomass returned but not biomass harvested treatment. A significant response to temperature was also observed in response to biomass return, but only in the no-till treatment. When probing these variables at the OTU level, 90% of OTUs influenced by moisture and 98% of OTUs influenced by temperature showed a decrease in relative abundance in response to increasing temperature or moisture. Many of these OTUs had high relative abundances in the fall or spring months (Supplemental Figures 3.12, 3.13).

Rainfall and temperature both peak during the growing season (June – August) and are lower at the beginning and end of the growing season (April, October – November) (Supplemental Figure 3.2). Thus, the response of fungal OTUs could be

associated with plant biomass inputs, which occurs in mid-late October, and which is correlated with decreases in temperature and moisture. To add support to this hypothesis, we examined abundance of fungal OTUs in November as compared with August. 21 OTUs from the NTR treatment specifically showed enrichment in November, with abundances enriched from 5 - to 990 - fold. Additionally, 71% of these OTUs were the same OTUs that responded to temperature in the NTR treatment. Four OTUs increased in abundance over 100-fold: OTU.2624 (genus *Mrakiella*), OTU.663 (genus *Articulospora*), OTU.549 (increase of 1,040-fold, genus unknown, family *Helotiaceae*), and OTU.64249 (genus unknown, class *Dothideomycetes*). Members of *Mrakiella* are unicellular yeasts and have been documented to grow on cellobiose, xylose, arabinose, soluble starch, and have previously been found in soils (Fell and Margesin, 2011). However, little is known about their ecology in soils. *Articulospora* is a genus with members known to participate in litter decomposition in the aquatic environment (Nikolcheva et al., 2005).

Thus, in this study, temperature and moisture may be correlated with other seasonal variables, such as change in carbon flow (root exudates, biomass management) that may influence community composition. The carbon input of corn plant biomass in the biomass returned treatment may drive seasonal changes in the fungal community. We see a similar phenomenon in the bacterial community (see chapter 2), with the bacterial community of the NTR treatment differentiating from communities of other treatments in the late fall and early spring, after biomass application.

Previous work has found evidence for shifts in soil bacterial communities upon plant

biomass addition (Lauber et al., 2013; Lipson, 2007; Pascault et al., 2013; Pfeiffer et al., 2013; Whitman et al., 2016). Additionally, the corn biomass added to soils in the fall is a complex mixture of carbon, differing largely from the simple C input of rhizodeposition during parts of the growing season. In a study looking at AMF, seasonal changes in host-plant carbon flow were hypothesized to account for differences in the community from summer to winter (Dumbrell et al., 2011). Further, other studies have proposed reduction of root exudate as a driver of microbial community composition in the fall (Griffiths et al., 2003; Stevenson et al., 2014; Thoms and Gleixner, 2013; Whitman et al., 2016).

### ***3.5.3 Change of Abundance of Specific OTUs Contributes to Seasonal Community Change***

Having high-throughput sequencing data allows for identification of specific OTUs affected by these sources of variation. We tested differential abundance of OTUs within each treatment over the continuous temperature range observed in this study. Differentially enriched OTUs primarily came from soils under NTR treatment and decreased in abundance with increasing temperature. This is in agreement with our results from correlation of community dissimilarity and difference in temperature and moisture, and also correlates with seasonal biomass inputs.

As temperature and moisture may co-vary with biomass application in this situation, we can hypothesize that OTUs whose abundance negatively correlates with temperature may also increase in abundance with biomass application, or a

combination of the two. The fungal class with the most OTUs influenced by temperature is the *Dothideomycetes*, including orders *Pleosporales*, *Capnodiales*, with some orders not able to be annotated (none were able to be classified at the genus level).

*Tremellomycetes* (genera *Dioszegia*, *Bullera*, *Hannaella*, *Cryptococcus*, *Mrakiella*, and unidentified) also has several responders to temperature. OTUs responding to moisture were mainly identified as *Sordariomycetes* (genera *Fusarium*, *Trichoderma*, and *Myrmecridium*), *Leotiomyces* (genera *Phialocephala*, *Hymenoscyphus*), or *Dothideomyces* (genus *Drechslera*). OTU.549, an unidentified *Tremellomycetes*, which responded in all treatments to temperature and in PTH to moisture, was found to increase relative abundance 1,040 –fold from August to November. We also identified known plant pathogens, such as *Fusarium* (2 OTUs) *Drechslera* (1 OTU), *Leptosphaeria* (1 OTU), and *Microdochium* (1 OTU) that increased 2 – 21-fold in abundance in November or April.

Few moisture and temperature responsive OTUs were found in more than one treatment. Thus, it seems that each treatment has unique OTUs that change over time. Also, only five OTUs were identified whose abundance correlated with changes to both moisture and temperature. This result suggests that the seasonal changes in microbial community composition that we see may result from specific interactions between temperature, moisture, carbon inputs, and other abiotic variables that we did not measure.



#### **3.5.4 OTUs Differentially Enriched by Land Management are Enriched under No-Till and Biomass Returned Conditions**

We also identified OTUs whose abundance was affected by either tillage, biomass management, or both land managements. The majority of *Basidiomycota* OTUs are enriched under no-till, biomass returned conditions. Many *Basidiomycetes* are filamentous fungi with hyphal structures, hence these fungi may not be favored in tilled fields. Previous work has identified a greater abundance of russuloid *Basidiomycetes* (quantified by ELISA) in no-till soils than till soils (Caesar-TonThat et al., 2010). Additionally, the no-till returned biomass conditions of the field site contain significantly more carbon (1.3 to 1.8-fold more than till) to support fungal growth. 85% of OTUs affected by biomass management demonstrate significant enrichment in returned biomass conditions.

Even within the same genus of fungi, a diversity of habitats in relation to soil horizon or plant community exist (Taylor et al., 2013). We see a similar result in terms of tillage for orders within the phylum *Ascomycota*. Only a few orders (*Pleosporales*, *Hypocreales*, *Saccharomycetales*, *Xylariales*, *Chaetothyriales*, *Capnodiales*) show a strong preference for differential enrichment in either till or no-till conditions, while most contain some OTUs enriched in till conditions and some enriched in no-till. This suggests that, for most *Ascomycetes*, differential enrichment in tillage conditions is not conserved at phylogenetic levels of order or higher.

### 3.6 Conclusion

We show that a variety of factors influence fungal community composition. Notably, tillage and biomass management impact fungal communities with distinct interactions across time and seasonal variables. Specifically, no-till soils with returned biomass support a fungal community that differs from other treatments in the fall and spring seasons, but becomes more similar in the summer. It is important to understand what members of the fungal community might be affected by land management, time, and the interactions between them in order to predict change in communities with future environmental change. Additionally, predicting these changes is important as they may result in changes in soil carbon dynamics, especially when they affect entire phylum of fungi, such as the reduced presence of *Basidiomycota* but continued presence of *Ascomycota* in tilled soils.

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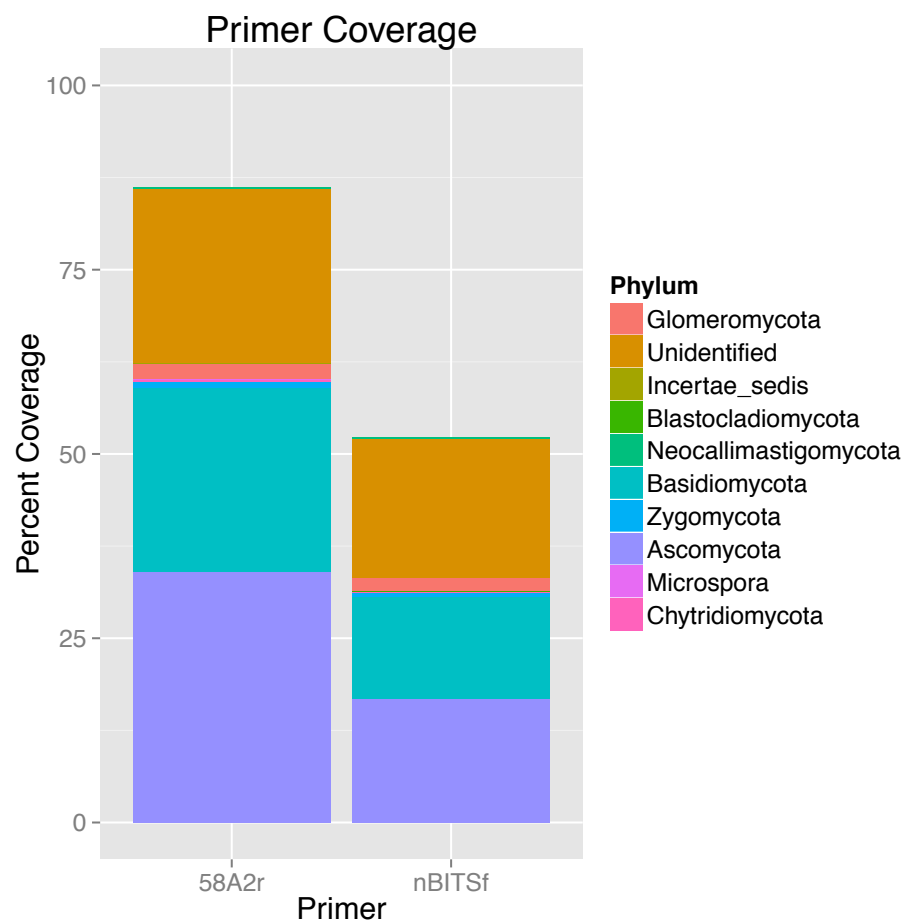


### 3.8 Supplemental Figures and Tables

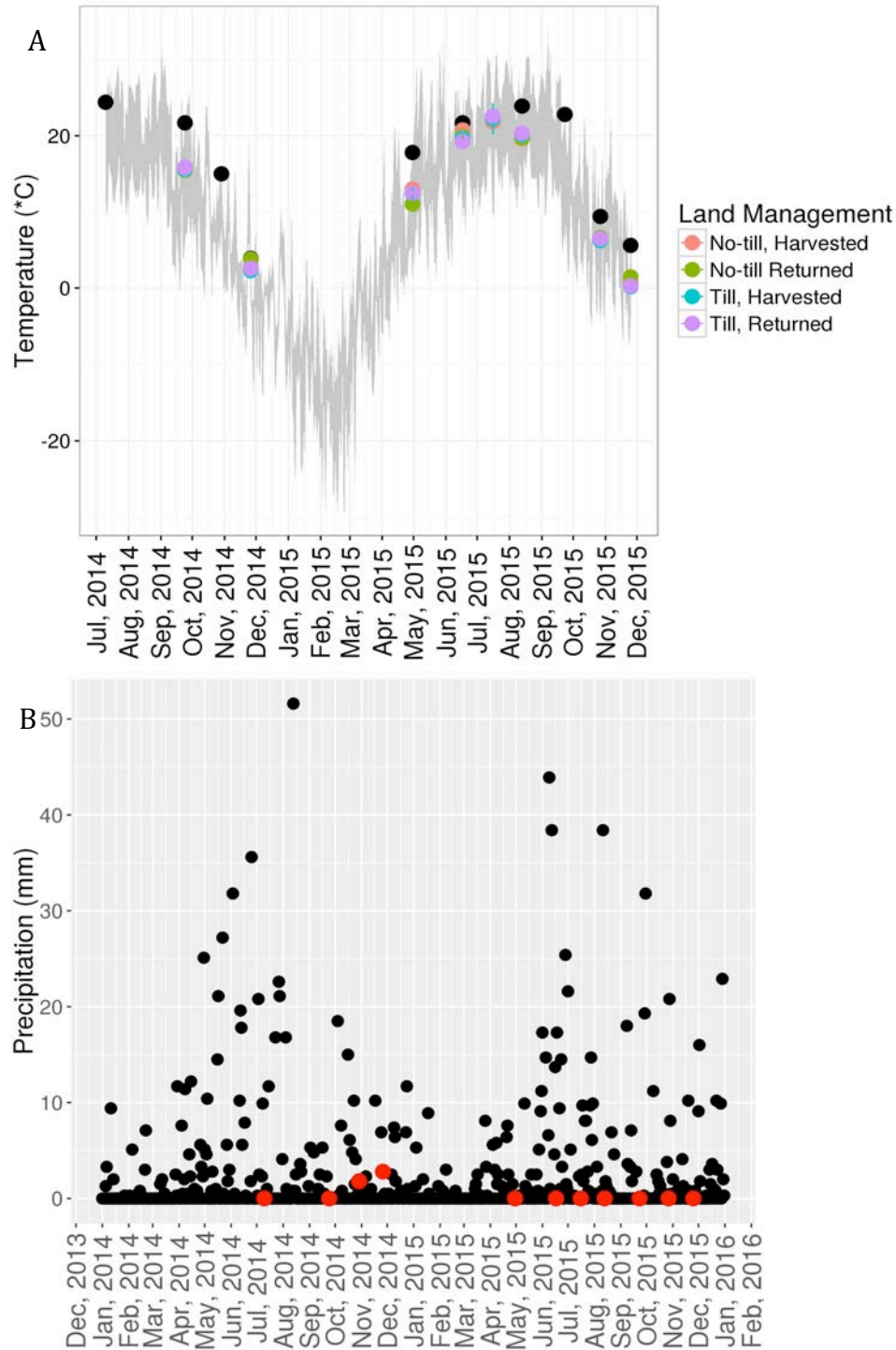
**Supplemental Table 3.1.** ITS primers tested using *in silico* Primer Prospector analysis.

Primer Name	Sequence (5' to 3')	Reference	Region
B58S3r	GAGATCCRTTGYTRAAAGTT	Bokulich and Mills (2013)	5.8S Reverse
NS1lf	GATTGAATGGCTTAGTGAGG	Martin & Rygiewicz (2005)	SSU Forward
ITS4r_KYO3	CTBTTCVCKCTTCACTCG	Toju et al (2012)	LSU reverse
ITS1-F_KYO2	TAGAGGAAGTAAAAGTCGTAA	Toju et al (2012)	SSU Forward
ITS4_KYO2	RBTTTCTTTTCCTCCGCT	Toju et al (2012)	LSU Reverse
ITS3_KYO2	GATGAAGAACYAGYRAA	Toju et al (2012)	5.8S Forward
ITS2_KYO2	TTYRCTRGGTTCTTCATC	Toju et al (2012)	5.8S Reverse
ITS4_KYO1	TCCTCCGCTTWTGWTGTC	Toju et al (2012)	LSU Reverse
ITS3_KYO1	AHCGATGAAGAACRYAG	Toju et al (2012)	5.8S Forward
ITS2_KYO1	CTRYGTTCTTCATCGDT	Toju et al (2012)	5.8S Reverse
ITS1-F_KYO1	CTHGGTCATTAGAGGAATAA	Toju et al (2012)	SSU Forward
BITS2f	ACCTGCGGARGGATCA	Bokulich and Mills (2013)	SSU Forward
flITS9f	GAACGCAGCRAANNNGYGA	Ihrmark et al. (2012)	5.8S Forward
flITS7f	GTGARTCATCGAATCTTTG	Ihrmark et al. (2012)	5.8S Forward
ITS5f	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)	SSU Forward
ITS4r	TCCTCCGCTTATTGATATGC	White et al. (1990)	LSU Reverse
ITS3f	GCATCGATGAAGAACGCAGC	White et al. (1990)	5.8S Forward
ITS2r	GCTGCGTTCTTCATCGATGC	White et al. (1990)	5.8S Reverse
nBITS2f	RCCWGYGGRRGGATCA (modified from BITS2f)	Bokulich and Mills (2013)	SSU Forward
58A2r	CTGCGTTCTTCATCGAT	Martin & Rygiewicz (2005)	5.8S Forward

(Bokulich and Mills, 2013; Ihrmark et al., 2012; Martin and Rygiewicz, 2005; Toju et al., 2012)



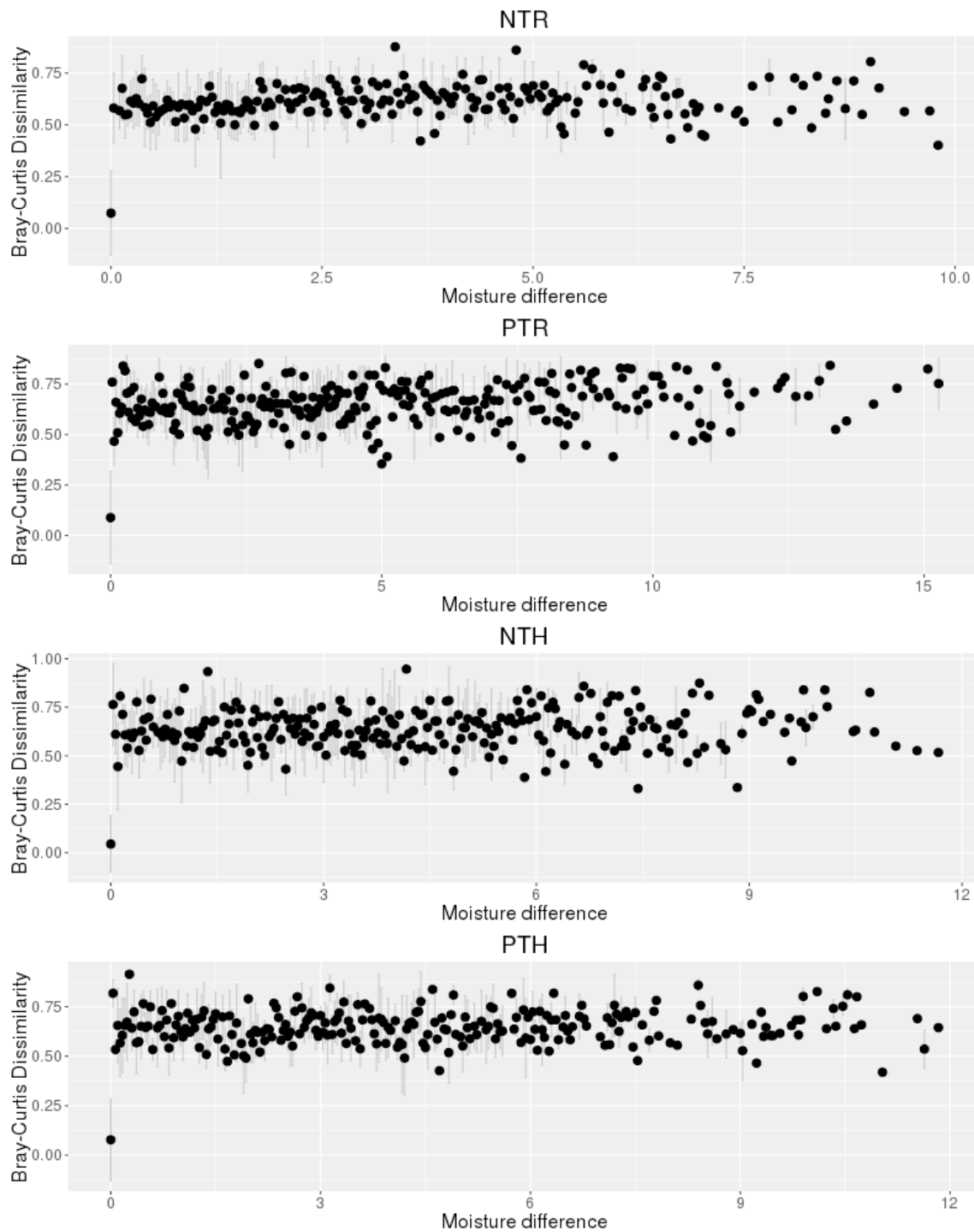
**Supplemental Figure 3.1.** Percent coverage of forward and reverse ITS primers used in this study on the representative set of fungal ITS sequences of the UNITE database (version 7). Barplots are colored by phylum.



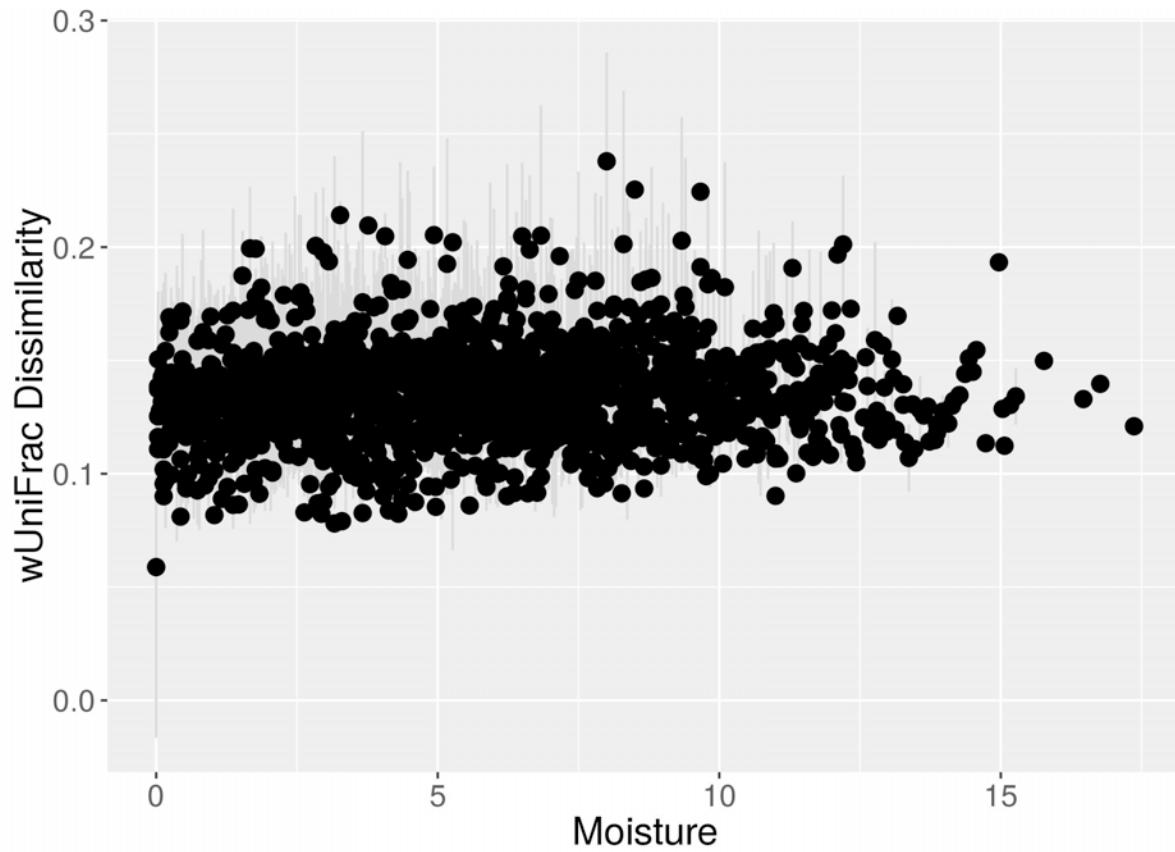
**Supplemental Figure 3.2. (A)** Ambient air temperature range over sampling time period. Black points represent maximum air temperatures on sampling days. Colored points represent soil temperatures for each land management on subset of sampling days (July 2014, October 2014, and September 2015 excluded) **(B)** Precipitation values (mm) for the sampling time period. Red dots indicate precipitation levels on sampling days.

**Supplemental Table 3.2.** Chao1 richness values and Pielou's Evenness values for each treatment, averaged over all sampling time points. Values with the same superscript are not significantly different from another at  $p < 0.05$  (Tukey's HSD).

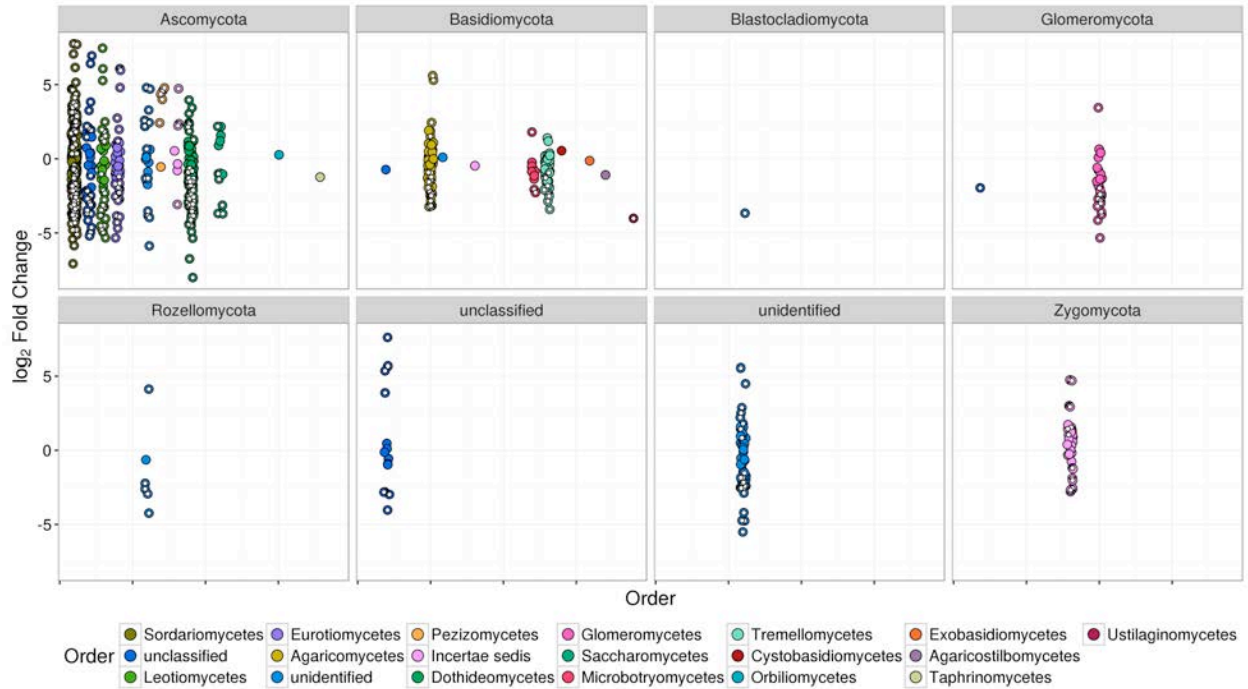
Treatment	Chao1 Richness	Pielou's Evenness
NTR	$302.4 \pm 24.7^a$	$0.73 \pm 0.1^a$
NTH	$315.5 \pm 19.3^a$	$0.72 \pm 0.1^a$
PTH	$272.7 \pm 19.6^{ab}$	$0.70 \pm 0.1^{ab}$
PTR	$257.4 \pm 18.5^b$	$0.67 \pm 0.1^b$



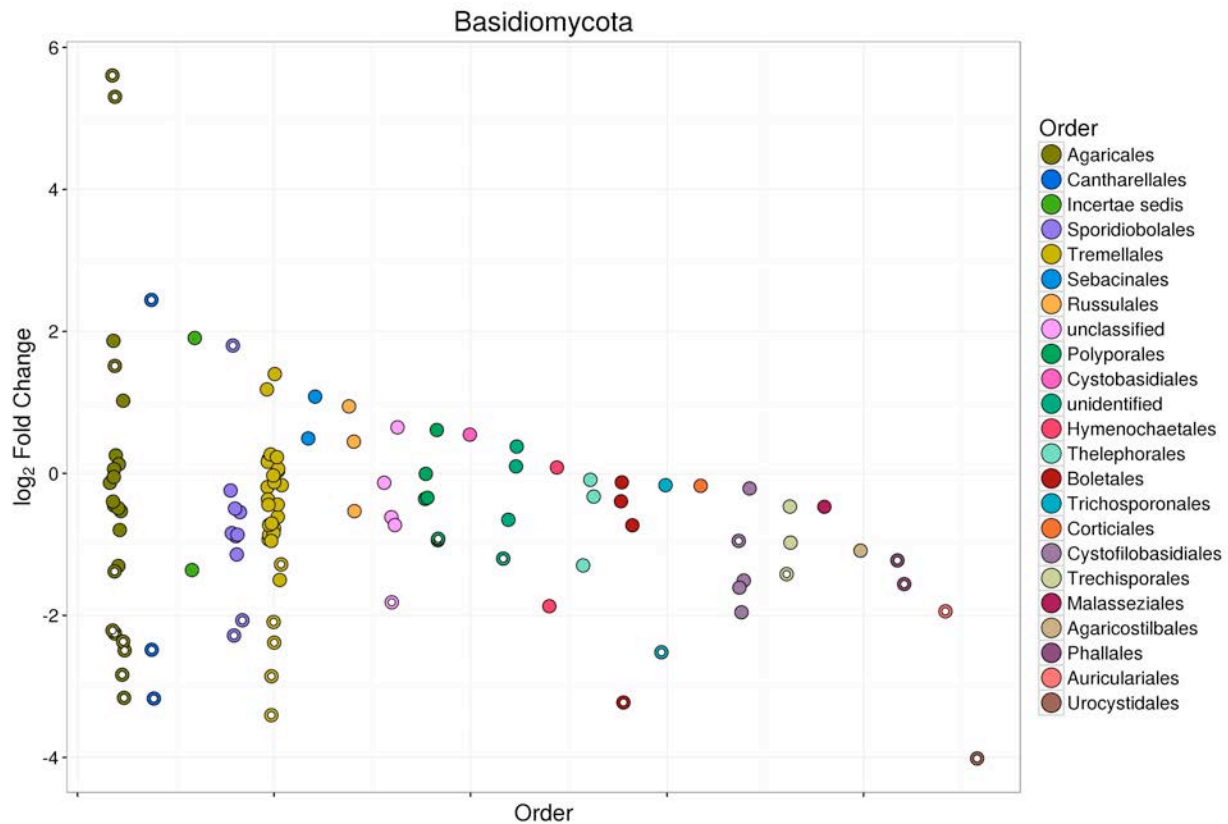
**Supplemental Figure 3.3.** Bray-Curtis dissimilarity pairwise distances vs. pairwise moisture distances for samples within the same land management. Both NTR and PTR treatment shows a significant correlation between community dissimilarity and temperature (Mantel test,  $p = 0.04$ ,  $R^2 = 0.14$ ;  $p = 0.02$ ,  $R^2 = 0.18$ ).



**Supplemental Figure 3.4.** Weighted UniFrac dissimilarity pairwise distances vs. pairwise moisture distances for bacterial samples.

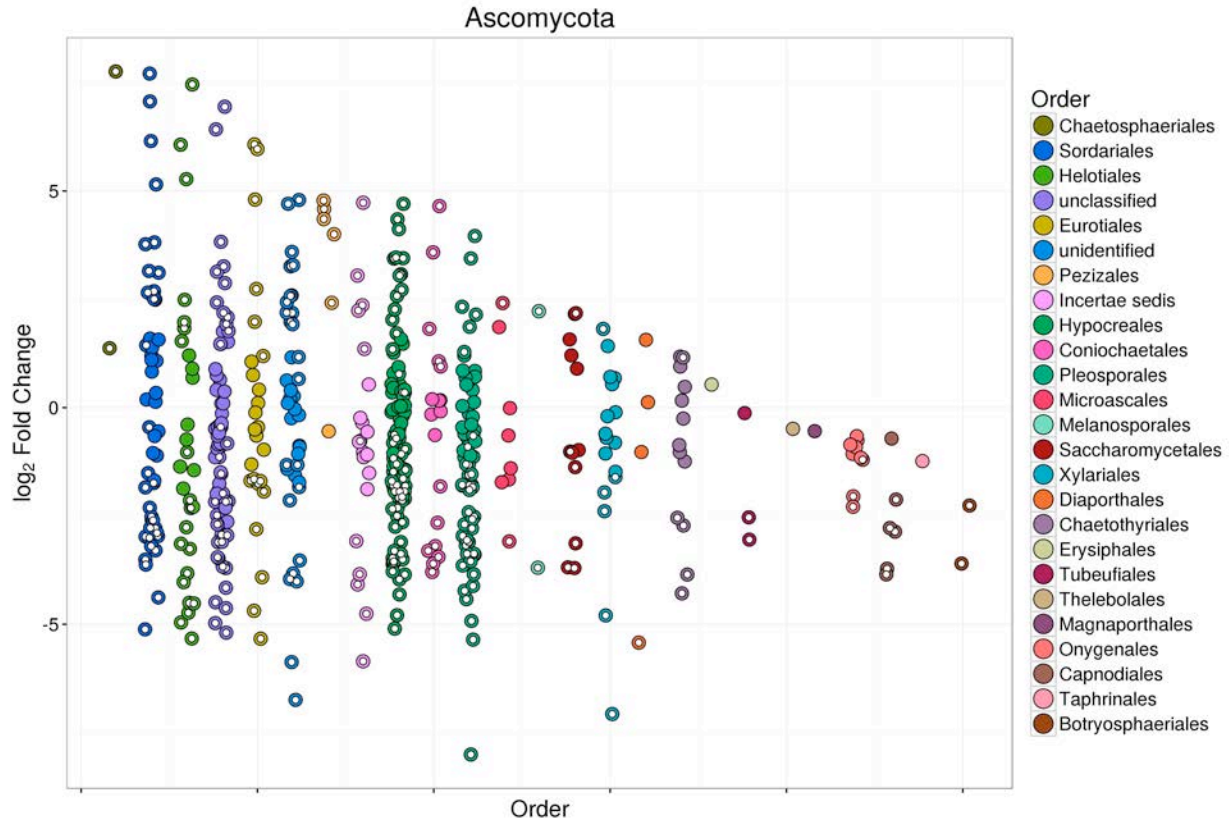


**Supplemental Figure 3.5.** Log<sub>2</sub>-fold change of OTU abundance in till (positive values) vs no till treatment (negative values) arranged by fungal order. Colors represent fungal order. Log<sub>2</sub>-fold change are faceted by phylum and significantly enriched OTUs are identified by a white dot within the colored circle.

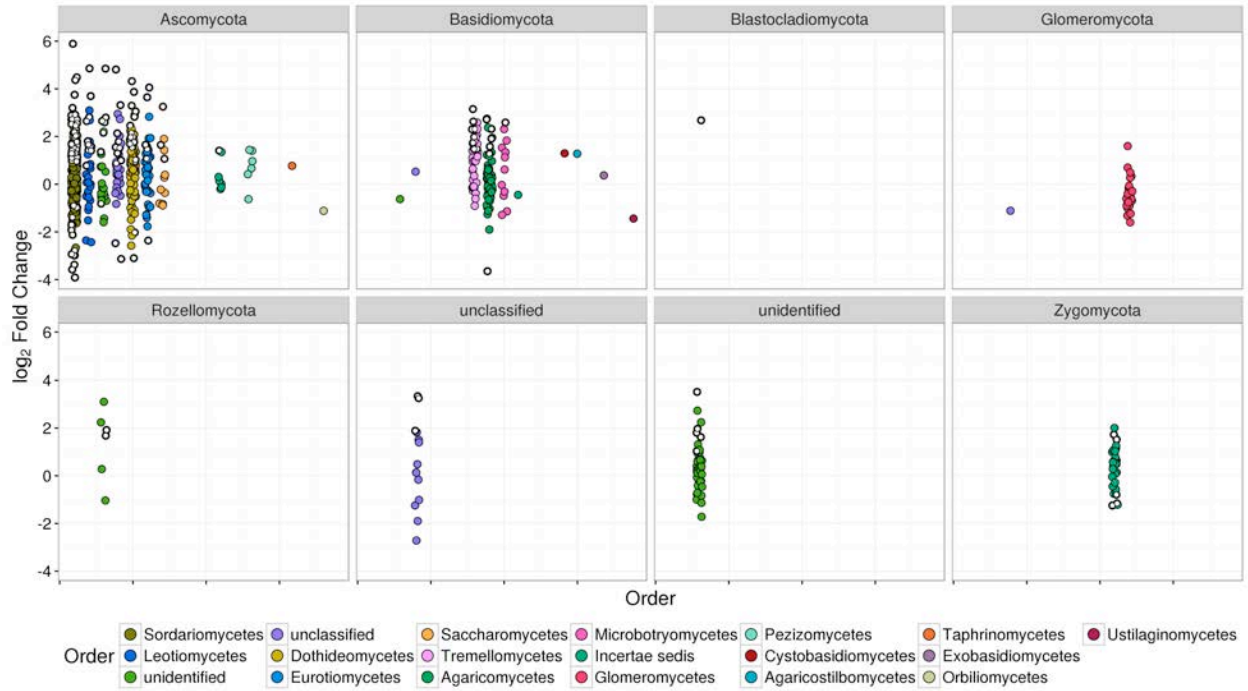


**Supplementary Figure 3.6.** Log<sub>2</sub>-fold change of OTU abundance in till (positive values) vs no till treatment (negative values) arranged by fungal order for all OTUs annotated as Basidiomycetes. Colors represent fungal order. Significantly enriched OTUs are identified by a white dot within the colored circle.

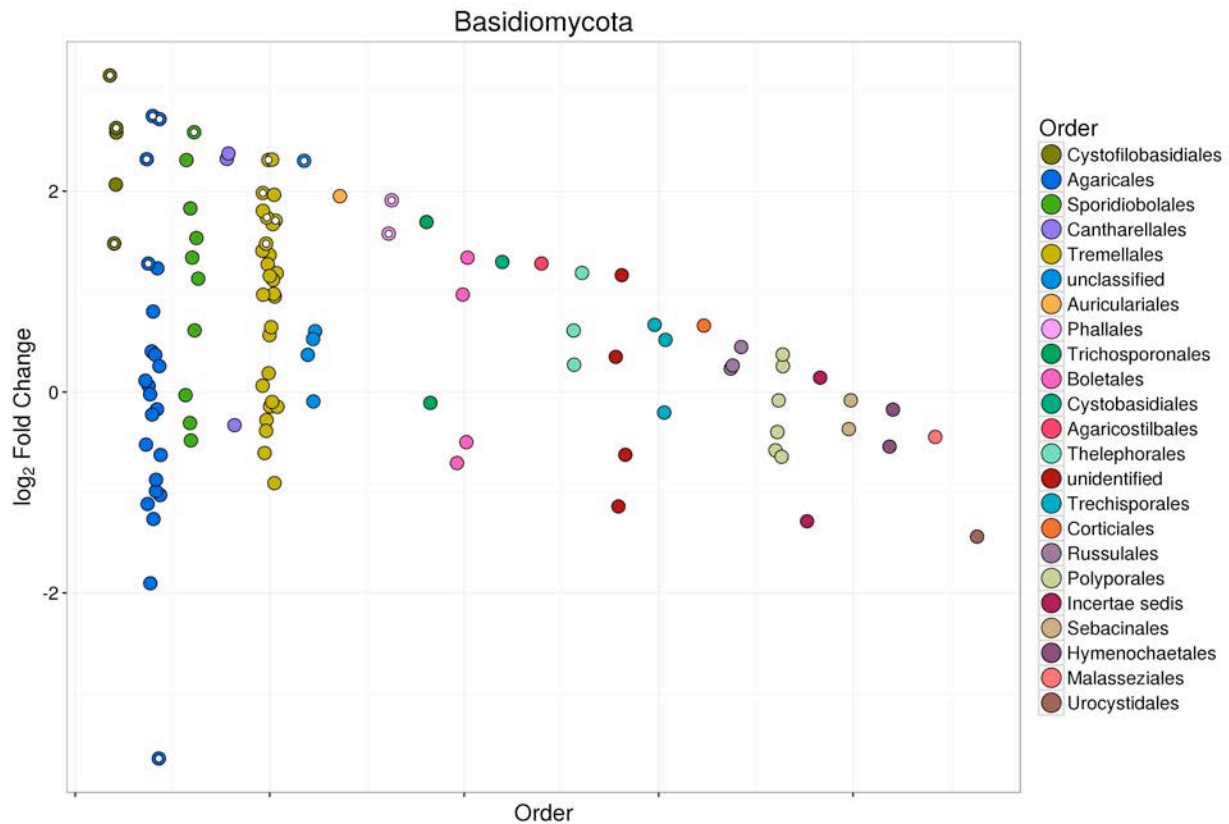




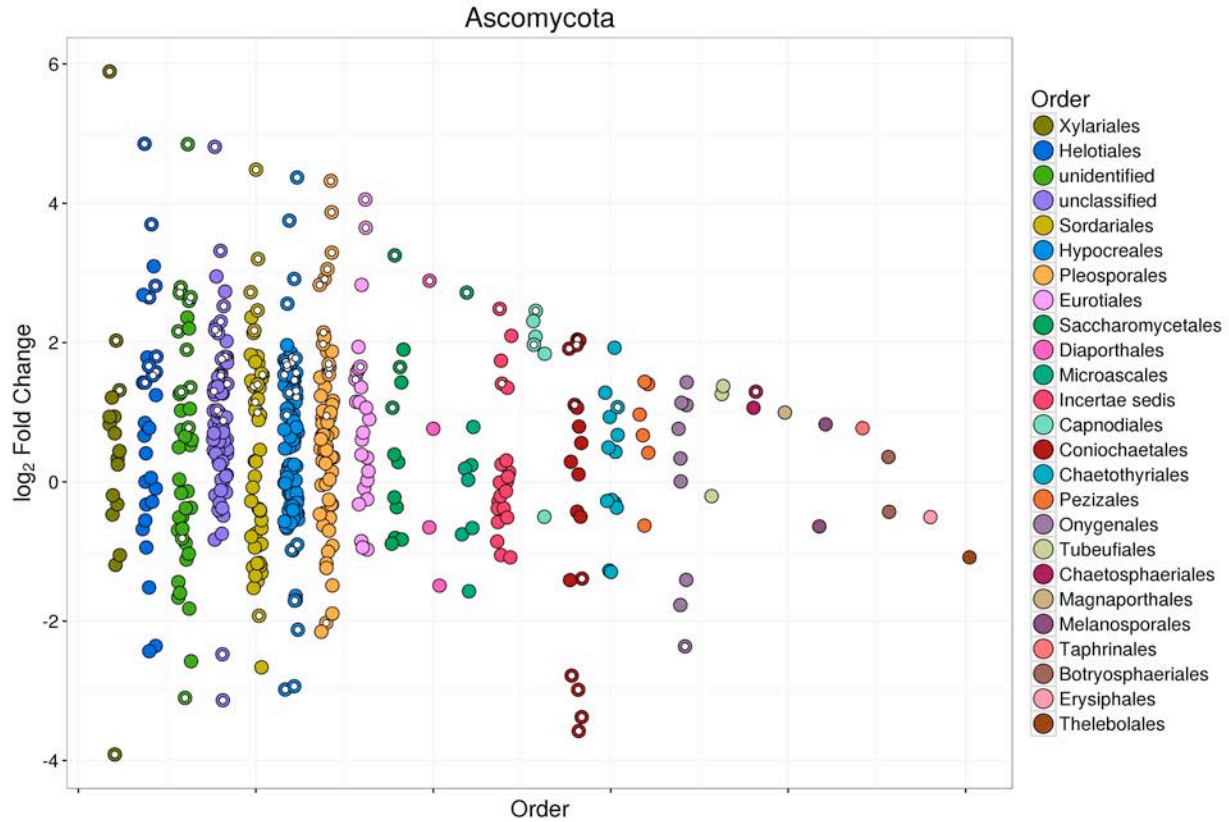
**Supplemental Figure 3.7.** Log<sub>2</sub>-fold change of OTU abundance in till (positive values) vs no till treatment (negative values) arranged by fungal order for all OTUs annotated as Ascomycetes. Colors represent fungal order. Significantly enriched OTUs are identified by a white dot within the colored circle.



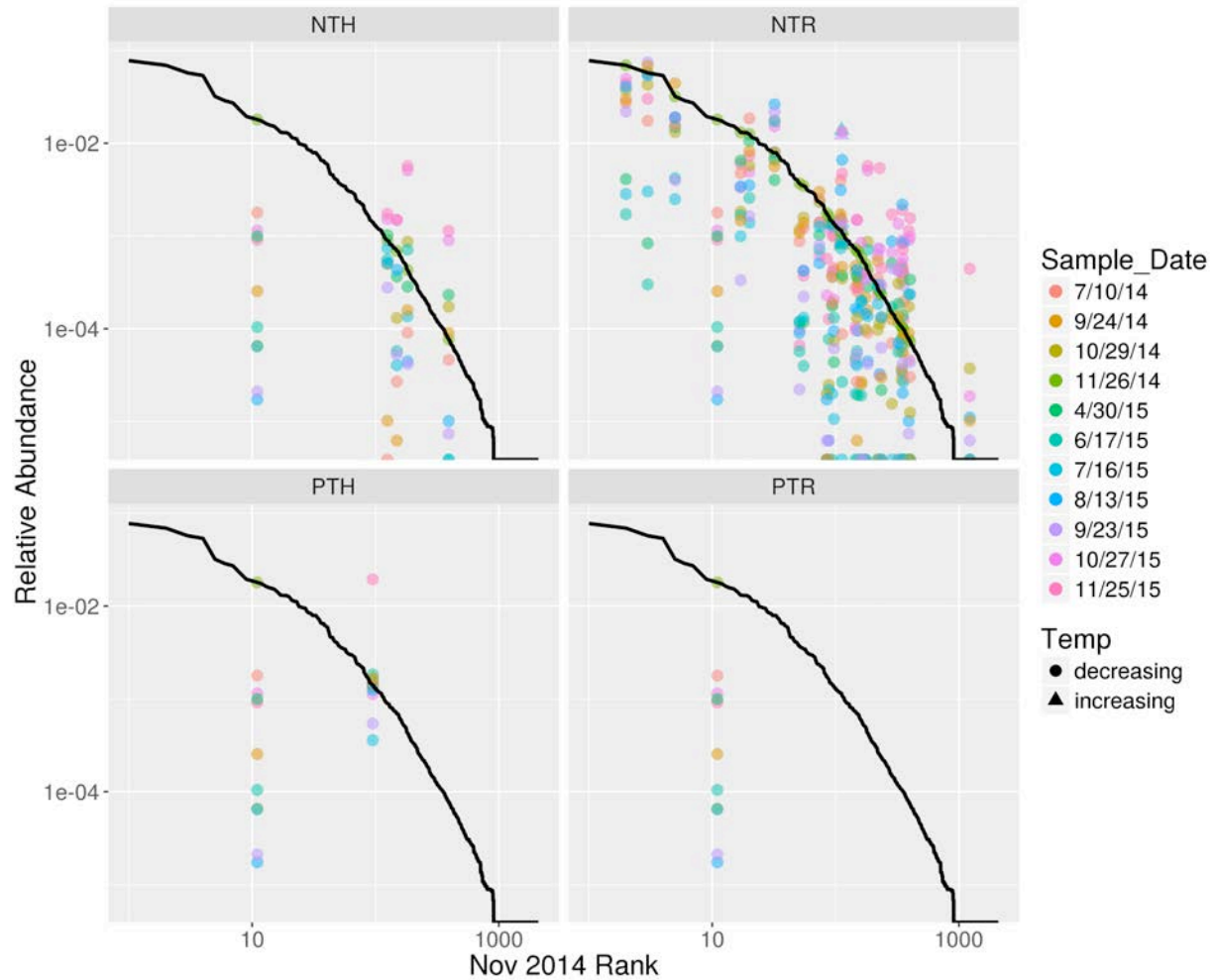
**Supplemental Figure 3.8.** Log<sub>2</sub>-fold change of OTU abundance in returned (positive values) vs harvested biomass treatment (negative values) arranged by fungal order. Colors represent fungal order. Log<sub>2</sub>-fold change are faceted by phylum and significantly enriched OTUs are identified by a black dot within the colored circle.



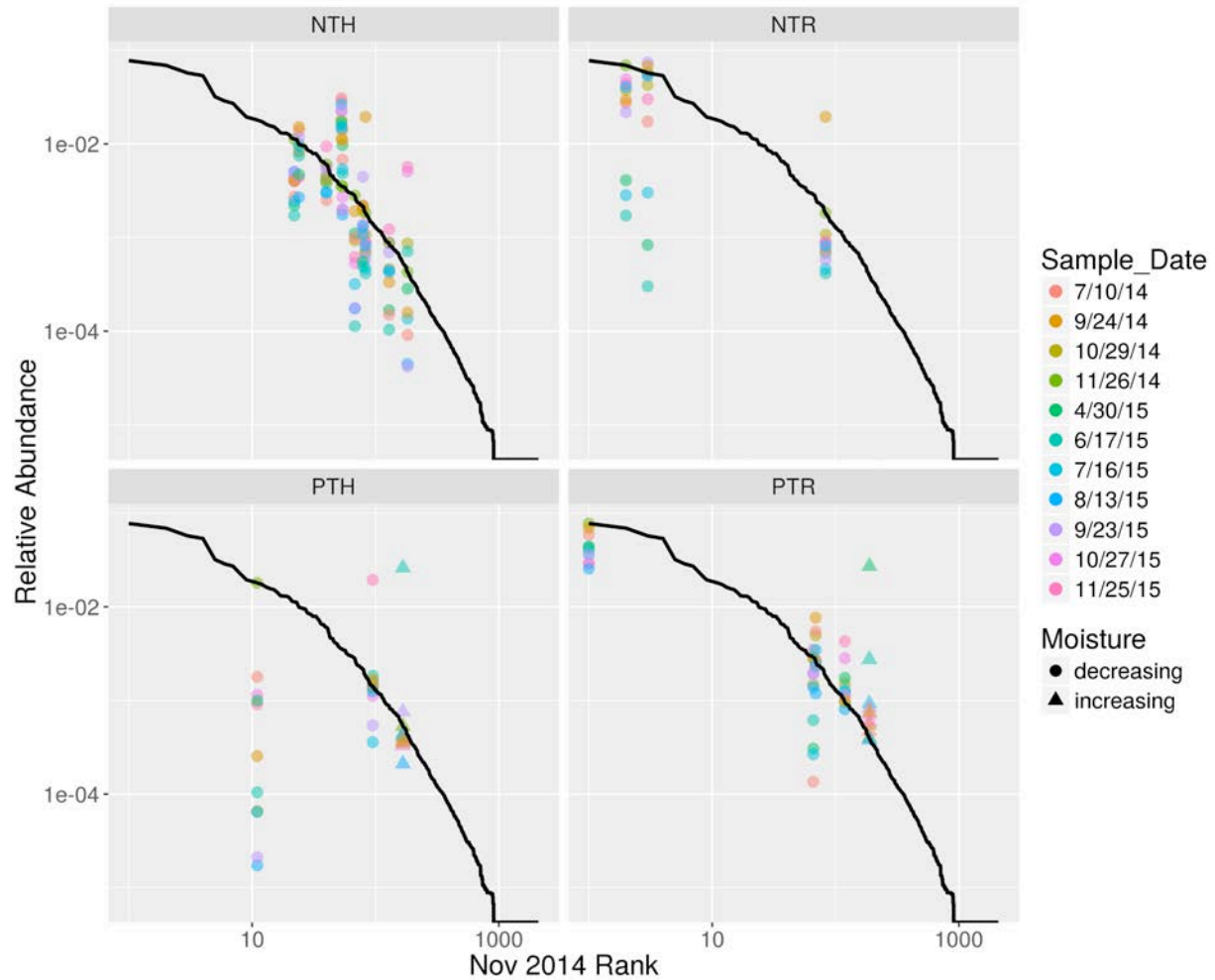
**Supplemental Figure 3.9.** Log<sub>2</sub>-fold change of OTU abundance in returned (positive values) vs harvested biomass treatment (negative values) arranged by fungal order for all OTUs annotated as Basidiomycetes. Colors represent fungal order. Significantly enriched OTUs are identified by a black dot within the colored circle.



**Supplemental Figure 3.10.** Log<sub>2</sub>-fold change of OTU abundance in returned (positive values) vs harvested biomass treatment (negative values) arranged by fungal order for all OTUs annotated as Ascomycetes. Colors represent fungal order. Significantly enriched OTUs are identified by a black dot within the colored circle.



**Supplemental Figure 3.11.** November 2014 rank abundance of OTUs whose abundance significantly correlated with temperature (see Figure 3.5a). Different colored points represent the abundance of that OTU at different sampling dates and shapes indicate whether correlation between abundance and temperature is negative (circle) or positive (triangle). The figure is faceted by land management.



**Supplemental Figure 3.12.** November 2014 rank abundance of OTUs whose abundance significantly correlated with moisture (see Figure 3.5b). Different colored points represent the abundance of that OTU at different sampling dates and shapes indicate whether correlation between abundance and temperature is negative (circle) or positive (triangle). The figure is faceted by land management.

## **4 Chapter 4 : Structural Differences in Bacterial Community Composition Contribute to Differences in Community Function in Cellulose and Xylose Cycling**

### **4.1 Abstract**

Bacteria are essential to the cycling and storage of carbon in the soil ecosystem. Tillage decreases soil organic matter content and changes the composition of soil microbial communities. However, little is known about the functionality of bacterial community in terms of carbon degradation in no-till vs tilled soils and whether these differences in biological function may contribute to differences in organic matter loss pathways. Microbial contributions to the degradation of both dissolved and particulate carbon compounds were contrasted in no-till and tilled soils by using high resolution DNA stable isotope probing (HR-SIP) to evaluate the temporal dynamics of  $^{13}\text{C}$ -xylose and  $^{13}\text{C}$ -cellulose assimilation over 30 days in a set of soils from a long-term tillage experimental in Chazy, NY. HR-SIP was combined with GC-MS measurement of  $^{13}\text{CO}_2$  production. We find that no-till soil has significantly higher rates of  $^{13}\text{C}$ -xylose and  $^{13}\text{C}$ -cellulose, mineralization relative to tilled soil. The bacteria that incorporate  $^{13}\text{C}$  xylose differ in tilled vs. no-till soils at the beginning of the experiment (days 1,3) but became more similar over time. Bacteria that incorporate  $^{13}\text{C}$  cellulose also differ across tillage at the beginning of the experiment (days 3 and 7) and become more similar over time, but at a smaller magnitude. Comparing the incorporation of  $^{13}\text{C}$  from cellulose and xylose into the bacterial community in tilled vs. no-till soils shows that the bacteria participating in carbon transformation differ as a function of soil management history, with

implications for carbon fate. The diversity of bacteria that incorporate xylose and cellulose, and rates of xylose and cellulose respiration varied with respect to tillage. These results suggest that changes in the structure of the microbial community affect both xylose and cellulose degradation.

## **4.2 Introduction**

Soil contains 2,500 Pg of carbon, a pool larger than the combined amount of carbon in the atmosphere and all plant life on earth (Chapin et al., 2011). Soil also contains an immense diversity of bacteria that use carbon substrates for energy or for transformation into biomass (Gans et al., 2005; Schloss et al., 2016; Torsvik et al., 1990; Tringe et al., 2005). It is thought that bacteria and other microbes account for ~90% of carbon cycling in soils (Swift, 1979) and that the inclusion of microbial parameters, such as biomass and community composition, improve models that predict carbon fluxes from soils (Graham et al., 2016; Powell et al., 2015; Treseder et al., 2012; Wieder et al., 2013).

Soil carbon dynamics differ with land management (Davidson and Ackerman, 1993). Agricultural management choices, especially those regarding tillage, can have large effects on soil carbon cycling (Buchanan and King, 1992; Franzluebbers et al., 1995). A majority of studies show that conventional tillage negatively impacts soil carbon stocks, reduces aggregation, and contributes to erosion (Govaerts et al., 2009; Turmel et al., 2015). No-till management, however, shows evidence of contributing to increases in soil carbon pools, increasing aggregation, and reduction in erosion



(Govaerts et al., 2009).

Tillage also impacts microbial communities, with distinct communities found in till vs. no-till soils (see Chapter 2, Babujia et al., 2010; Drijber et al., 2000; Frey et al., 1999; Lupwayi et al., 2001; Mbuthia et al., 2015; Navarro-Noya et al., 2013; Ramirez-Villanueva et al., 2015; Spedding et al., 2004). No-till soils contain greater levels of markers associated with *Actinobacteria* and mycorrhizal fungi (Drijber et al., 2000; Mbuthia et al., 2015; Navarro-Noya et al., 2013), while evidence exists for *Acidobacteria*, *Armatimonadetes*, *Gemmatimonadetes*, and *Chloroflexi* enrichment in tilled soils (Jiménez-Bueno et al., 2016). As microbial community composition has been linked to regulation of decomposition (Graham et al., 2016; Strickland et al., 2009; Treseder et al., 2012; Waldrop and Firestone, 2006; Waldrop et al., 2000), it is possible that changes in bacterial community due to tillage may contribute to functional changes in soil carbon cycling.

Evidence connecting changes in bacterial community composition to differences in function has been found in the soil environment for several processes including nitrogen fixation (Hsu and Buckley, 2008), denitrification (Cavigelli and Robertson, 2000), nitrification (Hawkes et al., 2005), response to global change stresses (increased CO<sub>2</sub> and temperature, Amend et al., 2015), and carbon cycling (Fanin et al., 2015; Graham et al., 2016; McGuire and Treseder, 2010; Strickland et al., 2009; Waldrop and Firestone, 2006). However, claims for functional redundancy of bacterial communities also exist, specifically for processes in carbon cycling (Rousk et al., 2009; Souza et al., 2015). Additionally, microbial community composition may be decoupled from function

in certain circumstances, due to specific rate-limiting processes (Schimel and Schaeffer, 2012), microbial dormancy (Jones and Lennon, 2010), or horizontal gene transfer (Smets and Barkay, 2005). In other cases, abiotic factors, such as moisture, temperature, pH, soil chemistry and structure, or substrate quality influence function (Blazewicz et al., 2013; Cleveland et al.; Davidson and Janssens, 2006; Fanin et al., 2015; Fierer and Schimel, 2002; Rousk et al., 2010; Schimel and Schaeffer, 2012).

Although differences in microbial community structure in till and no-till soils have been hypothesized to account for differences in carbon cycling in these soils, there has been little evidence presented linking community structure and function. High resolution stable isotope probing (HR-SIP) is a technique that allows the identification of specific OTUs involved in the cycling of carbon substrates. In HR-SIP, a substrate labeled with heavy isotope (ie.  $^{13}\text{C}$ ) is added to soil and becomes incorporated into microbial biomass (ie. DNA) over time. During this incubation, we can track production of  $^{13}\text{CO}_2$ . At different time points during incubation, we extract DNA of the microbial community from the  $^{13}\text{C}$  treatment, as well as the soil from a treatment receiving the substrate labeled with the natural abundance isotope (ie.  $^{12}\text{C}$ ). These sets of DNA are then individually centrifuged to separate high density DNA from low density DNA, and several fractions of DNA are collected, representing a range of isotopic incorporation into DNA. The SSU rRNA gene from each fraction is amplified and sequenced in a high-throughput manner. We can then compare bacterial communities from high-density fractions of the  $^{13}\text{C}$  treatment to high-density fractions of the  $^{12}\text{C}$  to identify specific communities members that have incorporated the isotopic substrate.

Using HR-SIP, we tested the impact of tillage on cycling of cellulose and xylose in agricultural soils from a long-term (42 year) tillage experimental field site to examine the relationship between bacterial structure and function. We tested against the null hypothesis that community structure (beta diversity) has no impact on function in carbon cycling. We assessed the production of  $^{13}\text{CO}_2$  between till and no-till soils for both  $^{13}\text{C}$ -cellulose and  $^{13}\text{C}$ -xylose substrates, and compared the bacterial communities involved in cellulose and xylose cycling between tillage managements. The results of this study provide insight into the link between bacterial community composition and the function of that community in carbon cycling : might differences in the bacterial community composition in till and no-till soils involved in carbon cycling impact the magnitude or rate of carbon cycling? Answering this question will provide further understanding of the role of microbial communities in carbon cycling and parameters that are important to include in development of soil carbon models.

## **4.3 Materials and Methods**

### ***4.3.1 Field Site and Soil Sampling***

We sampled soils from the long-term tillage research plot at the Miner Institute in Chazy, NY (Clinton County, 44°53.13'N, 73°28.40'W). The research plot is in a factorial 2 x 2 design, testing effects of both tillage and residue management. The plot contains four blocks, with each block contains four treatments: No-till, returned biomass (NTR); No-till, harvested biomass (NTH); Till, returned biomass (PTR); Till, harvested biomass (PTH).

Soils for stable isotope probing were sampled in September 2014 from all NTH and PTH plots. We collected 20 cores (5cm depth x 2.5 cm diameter) across each replicate plot (6 x 15.2 m). Cores from a unique replicate plot were combined and homogenized through sieving (2mm sieve). Soil was placed on ice during transport back to the lab, and stored at 4°C until the beginning of the experiment (3 days). We also collected soil moisture and temperature data for three equidistant points across plots, using moisture and temperature probes, and we averaged across the three points when analyzing data.

#### **4.3.2 Cellulose Production**

Both  $^{13}\text{C}$  and  $^{12}\text{C}$  Cellulose used in the stable isotope probing was prepared using *Gluconoacetobacter xylinus* following the method of Pepe Ranney and Campbell et al (Pepe-Ranney et al., 2016).

#### **4.3.3 Microcosm Set-Up**

Microcosms were set up in 250 mL Erlenmeyer flasks. 10g of dry weight soil were placed into microcosms with butyl rubber stoppers to prevent moisture evaporation. Dry weight was determined by gravimetric soil moisture measurements for each soil management and replicate (Berthrong et al., 2013). Microcosms were pre-incubated for 2 weeks until production of  $\text{CO}_2$  from the soil, as measured by GC-MS, stabilized following disruption through sieving and placement into microcosms. Throughout the experiment, stoppers were removed every two days and microcosms

were flushed with filtered (0.2  $\mu\text{m}$ ) air to exchange headspace air.

A total of 112 microcosms were prepared (Supplemental figure 4.1) which represent combinations of 2 field treatments (no-till and till), 4 field replicates, 4 amendment types ( $^{12}\text{C}$  control,  $^{13}\text{C}$ -xylose,  $^{13}\text{C}$ -cellulose, and  $\text{H}_2\text{O}$  control), and 1-5 sampling time points (5 time points for  $^{12}\text{C}$  control, 4 for  $^{13}\text{C}$  treatments, and 1 for  $\text{H}_2\text{O}$  control).

The five destructive sampling time points used for the  $^{12}\text{C}$ -control included 1 day, 3 days, 7 days, 14 days, and 30 days. For the  $^{13}\text{C}$ -xylose treatment, microcosms were destructively sampled at day 1, day 3, day 7, and day 14. The microcosms receiving  $^{13}\text{C}$ -cellulose treatment were harvested on day 3, day 7, day 14, and day 30.

We added carbon substrates to the microcosms in the form of a corn simulant. The simulant was based on the composition of corn stover (Huang et al., 2009) and contained cellulose (0.889  $\text{mg C g}^{-1}$  soil, 37.4% by mass), xylose (0.451  $\text{mg C g}^{-1}$  soil, 21.1% by mass), arabinose (0.062  $\text{mg C g}^{-1}$  soil, 2.9% by mass), mannose (0.034  $\text{mg C g}^{-1}$  soil, 1.6% by mass), galactose (0.042  $\text{mg C g}^{-1}$  soil, 2% by mass), and lignin (0.591  $\text{mg C g}^{-1}$  soil, 18% by mass). The remaining 18% mass was composed of amino acids (lab-made replica of Teknova Cat # C0705), and basal salt mixture (Murashige and Skoog, Sigma M5524). The simulant had a C:N ratio of 10, comparable to the C:N ratio of the soils used in the experiment. All components of the simulant, except for the cellulose and lignin, were added via liquid addition to 50% of the water holding capacity of the soils (calculated for each field replicate soil). This water holding capacity is thought to be optimal for microbial activity (D. M. Linn, 1984).

The  $^{12}\text{C}$  microcosm treatment contained carbon with natural abundance of  $^{13}\text{C}$ . The two  $^{13}\text{C}$  treatments substituted 99%  $^{13}\text{C}$  cellulose or xylose, respectively, instead of their  $^{12}\text{C}$  counterparts. The water control contained an equivalent amount of water and basal salts as added in the other treatments, to serve as a control for bottle and moisture effects.

Once microcosms were harvested, soils were stored at  $-80^{\circ}\text{C}$  until DNA extraction. Additionally, aliquots of day 30 soils were dried and prepared for isotopic analysis (UC Davis Stable Isotope Facility) and measurements of soil characteristics including pH, soil C, and soil N. We measured pH using standard 1:1 soil-water method, and total soil C and N were determined on oven dried and ground samples via LECO Treu Mac CN-2000\* elemental analyzer (LECO Instruments, Lansing, MI) as previously described (Berthrong et al., 2013)

#### **4.3.4 $\text{CO}_2$ Monitoring**

Throughout the 30-day course of the incubation,  $\text{CO}_2$  efflux from soils was measured via gas chromatography – mass spectrometry (GC-MS), using a Shimadzu QP2010S GCMS plumbed with Carboxen-1010 PLOT column (G001075, Supelco, St. Louis, MO). Microcosm headspace samples were injected into the GC, with an injection port temperature of  $200^{\circ}\text{C}$ . The oven temperature was held at  $35^{\circ}\text{C}$  for 7.5 minutes and then ramped to  $230^{\circ}\text{C}$  at a rate of  $40^{\circ}\text{C min}^{-1}$ . The column flow was  $1.58 \text{ mL min}^{-1}$  and the temperature at the GC-MS interface was  $230^{\circ}\text{C}$ . The quadrupole MS was run in selective ion mode (SIM), scanning for m/z of 44 ( $^{12}\text{CO}_2$ ), 45 ( $^{13}\text{CO}_2$ ), and a total ion

count (TIC). A CO<sub>2</sub> standard curve was run for each sample batch and used to quantify microcosm CO<sub>2</sub>.

#### ***4.3.5 DNA Extraction in Preparation for Isopycnic Centrifugation***

DNA from 4 x 0.25 g of soil from replicate 4 of all soil-treatment-date combinations, replicate 2 and 3 for xylose-day 3 samples of both soil types, and replicate 2 and 3 for cellulose-day 30 samples of both soil types was extracted using a modified Griffiths extraction (Griffiths et al., 2000). Briefly, cells were lysed by 1 minute of bead beating at 5.5 m s<sup>-1</sup> in 2 mL lysis tubes filled with 0.5 g of 0.1 mm diameter silica/zirconia beads (previously baked at 300°C for 4 hours to remove RNases), 0.5 mL extraction buffer (240 mM phosphate buffer with 0.5% N-lauryl sarcosyl), and 0.5 mL of phenol-chloroform-isoamyl alcohol (25:24:1). On completion of lysis, 85 µL of NaCl (5M) and 60 µL of a mixture of hexadecyltrimmonium bromide (CTAB, 10%) and NaCl (0.7M) were added to the tube. The tube was vortexed, chilled on ice for 1 minute, and centrifuged at 16,000 x g for 5 minutes at 4°C. The top, aqueous layer was pipetted into a new tube and placed on ice. The pellet was then re-extracted with 0.5 mL extraction buffer and 85 µL of NaCl (5M) and again centrifuged at 16,000 x g for 5 minutes at 4°C. The aqueous layer was again removed and added to the first aqueous layer. Combined aqueous layers were washed with 1 mL of chloroform : isoamyl alcohol (24:1). Nucleic acids were precipitated with 2 volumes of polyethylene glycol solution (30% PEG 8000, 1.6 M NaCl) on ice for 2 hours or at 4°C overnight. Precipitate was collected by centrifugation at 16,000 x g for 30 minutes at 4°C. Supernatant was removed and

pellets washed with 1 mL of cold 70% EtOH twice. Dried pellets were resuspended in 50  $\mu$ L TE and stored at -20°C.

For DNA molecules to reach equilibrium during isopycnic centrifugation, we selected for size of 4 – 14 kb via the Blue Pippin Prep machine (Sage Science; Beverly, MA). We used 0.75% agarose gel cassettes (Sage Science, BLF7510) and followed manufacturer's protocol.

#### ***4.3.6 DNA Extraction for Analysis of Unfractionated Samples***

All replicates of all isotope treatment – day – soil combinations (112 samples) were extracted using 2 x 0.25 g of soil and following the same altered Griffiths extraction as outlined above. Post-extraction, samples were cleaned using illustra™ MicroSpin™ G-50 columns (GE Healthcare; Buckinghamshire, UK; 27-5330-02) and magnetic bead purification (Agencourt AMPure XP purification; Beckman Coulter; Brea, CA; A63880), following manufacturer's protocol.

#### ***4.3.7 Isopycnic Centrifugation and Fractionation***

All samples from each isotope treatment – day – soil combination (26 total samples) of one replicate (from block # 4 of the field site) underwent isopycnic centrifugation. Additionally, two more sets of sample replicates (from blocks # 2 and 3 of the field site) underwent isopycnic centrifugation for the  $^{12}\text{C}$ -control and  $^{13}\text{C}$  – xylose, day 3 samples of both till and no-till soils (8 samples), as well as for the  $^{12}\text{C}$ -control and  $^{13}\text{C}$  – cellulose, day 30 samples of both till and no-till soils (8 samples). Thus, a total of



42 samples underwent centrifugation.

Isopycnic gradients were set up following Neufeld et al. (2007), with modifications (Neufeld et al., 2007). Briefly, for each sample, 6  $\mu\text{g}$  of DNA (in TE) was added to a cesium chloride (CsCl) density gradient solution ( $1.69 \text{ g mL}^{-1}$ ) in 4.7 mL polypropylene tube (Beckman Coulter; Brea, CA; 361621). The cesium chloride solution was made in a buffer containing 15 mM Tris-HCl, 15 mM EDTA, and 15 mM KCl, with a final pH of 8.0. Sample tubes were centrifuged at 55,000 rpm for 66+ h at  $20^\circ\text{C}$  on an Optima<sup>TM</sup> MAX-E ultracentrifuge (Beckman Coulter; Brea, CA) with a TLA-110 fixed-angle rotor.

After centrifugation, 100  $\mu\text{L}$  fractions were collected from the bottom of the tube by syringe pump-mediated water-displacement from the top of the tube at a rate of  $15 \mu\text{L s}^{-1}$  (Manefield et al., 2002). Fractions were collected in deep well 96-well plate (Corning, Tewksbury, MA; P-96-450V-C-S). Immediately after each fraction collection, refractive index ( $R_i$ ) was measured using a Reichart AR200 digital refractometer with modifications as described in Buckley et al. (2007).  $R_i$  was corrected for the  $R_i$  of the gradient buffer ( $R_{i \text{ corrected}} = R_{i \text{ observed}} - (R_{i \text{ buffer}} - R_{i \text{ water}})$ ). From the corrected  $R_i$ , buoyant density could be calculated:  $\rho = a\eta - b$ , where  $\rho$  is the density of the CsCl ( $\text{g mL}^{-1}$ ),  $\eta$  is the  $R_i$  corrected, and  $a$  and  $b$  are coefficient values of 10.9276 and 13.593, respectively, for CsCl at  $20^\circ\text{C}$  (Birnie, 1978).

Once collected, fractions were desalted and purified using Agencourt AMPure XP purification, following manufacturer's protocol (Beckman Coulter, Brea, CA; A63880). Quantification of purified DNA occurred using the Quant-IT PicoGreen dsDNA assay (Life Technologies, Grand Island, NY; P7589), using 2  $\mu\text{L}$  of each gradient fraction.

Fluorescence was measured using a FilterMax F5 plate reader (Molecular Devices, Sunnyvale, CA).

#### **4.3.8 *SSU rRNA Gene Library Preparation and Sequencing of Gradient Fractions***

After fractionation and desalting, fractions within the range of 1.673 – 1.774 g/mL were chosen for sequencing preparation. This density range represents DNA segments that range in GC content from 13.5% - 80% plus an additional 0.036 g/mL for maximum  $^{13}\text{C}$  labeling. An average number of 23 fractions per gradient were used, resulting in the preparation of 979 fractions for sequencing.

Primers 515f / 806r, with barcoding scheme as in Kozich et al (2013) were used for amplification of both gradient fractions and unfractionated samples. For gradient fractions, 25  $\mu\text{L}$  PCR reactions included 12.5  $\mu\text{L}$  Q5 High Fidelity Hot Start PCR Mastermix (New England Biolabs, M0494), 2.5  $\mu\text{L}$  of combined forward and reverse barcoded primer at 10  $\mu\text{M}$ , 0.625  $\mu\text{L}$  Picogreen reagent (4x concentration, used for visualization of PCR progress on qPCR machine; Life Technologies, Grand Island, NY; P7589 ), and 5 ng template DNA. PCRs done in triplicate and a Hamilton Microlab Starlet liquid handling robot was used for setting up PCR reactions.

Samples were concentrated down to 5 ng/ $\mu\text{L}$ , via vacuum centrifugation, and were sent to the Cornell Core Facility in Ithaca, NY to be run on an Illumina MiSeq, using the V2 chemistry with 2 x 250 bp read length.

#### **4.3.9 SSU rRNA Gene Library Preparation and Sequencing of Unfractionated Samples**

The same primers and barcoding scheme were used for 16s rRNA gene library preparation for unfractionated samples. Unfractionated samples include all substrate – day – and tillage management combinations, covering 112 samples. PCR set up was identical to fraction preparation, except for the addition of 1.25 µL of Bovine Serum Albumin (BSA, New England Biolabs; B9000s). PCRs were also run in triplicate and set up using the Hamilton Microlab Star liquid handling robot. Triplicate amplicons were pooled. Purification and normalization was done using the SequalPrep normalization kit (Invitrogen), using manufacturer’s protocol. Samples were concentrated down to 5 ng/uL, via vacuum centrifugation, and were sent to the Cornell Core Facility in Ithaca, NY to be run on an Illumina MiSeq, using the V2 chemistry with 2 x 250 bp read length.

#### **4.3.10 Post-Sequencing Analysis**

All code used for data processing and analysis can be found on Github (<https://github.com/chvtk/ChazySIP>).

##### *Data Processing for CO<sub>2</sub> fluxes*

We looked at CO<sub>2</sub> fluxes for cellulose and xylose separately, using a linear mixed effects model with tillage, day, and plot replicate as factors using R package “lme4” (Bates et al., 2013). We performed post-hoc, pairwise comparisons, using a Tukey’s p-value adjustment, between tillage treatments for individual days with the “lsmeans” R package (Lenth, 2016).

### *Sequencing Processing and Quality Control*

Raw Illumina sequencing reads were merged with PEAR, using program defaults, and then demultiplexed (Zhang et al., 2014). Illumina sequences were then screened by maximum expected errors, with reads exceeding a maximum expected error threshold of 1.0 being discarded. Alignment-based QC, using Mothur and a representative set from the Silva database (97% identity, version 111), was performed and sequences of lengths less than 253 bp and sequences with homopolymers greater than 8 bp were removed (Kozich et al., 2013).

### *OTU Clustering and Taxonomic Assignment*

The dataset (both fractionated and unfractionated samples) underwent OTU clustering using UParse (Edgar, 2013). Cluster seeds were identified with a non-redundant set of reads, using USearch version 7.0.1090, with a sequence identity threshold for a new OTU centroid set at 97%. With USearch/UParse, potential chimeras were identified during OTU centroid selection and could not become cluster centroids, eliminating chimeras from the read pool. All quality controlled reads of the combined dataset were then mapped to cluster centroids at an identity threshold of 97%, again using USearch. 67% of quality controlled reads were mapped to centroids. Unmapped reads did not count towards sample counts and were removed from downstream analyses. Following quality control and OTU binning, there were 49,009,806 sequences comprising of 25,178 OTUs across 1,350 samples (mean  $24,222 \pm 11,745$  sequences/sample).

Reads were taxonomically annotated using the UClust taxonomic annotation

framework in the QIIME software package with cluster seeds from the SILVA SSU rRNA representative set using 97% sequence identity OTUs as reference (release111) (Caporaso et al., 2010; Edgar, 2010; Pruesse et al., 2007). Reads annotated as “Chloroplast”, “Eukarya”, “Archaea”, “Unassigned” or “mitochondria” were removed from the dataset.

### *Bioinformatic Analyses*

R version 3.1.2 was used for all analyses, utilizing the phyloseq, vegan, dplyr, DESeq2, ggplot2, picante, and MetagenomeSeq libraries (Love et al., 2014; McMurdie and Holmes, 2013; R Core Team, 2014; Wickham and Chang, 2015; Wickham et al., 2015).

### *NMDS Ordination*

Weighted UniFrac used to calculate a phylogenetic distance matrix for samples (Lozupone et al., 2006). The distance matrix was visualized using Nonmetric Multidimensional Scaling (NMDS). Adonis statistics (Permutational Multivariate Analysis of Variance) were used to partition ordination variance attributed to isotope treatment, land management, and time for both unfractionated and fractionated samples.

### *Differential Expression Statistical Framework*

The differential expression statistical framework (DESeq2) was used to identify OTUs that were enriched in the  $^{13}\text{C}$  treatment as compared to the  $^{12}\text{C}$  treatment for both cellulose and xylose at individual time points of the experiment (Anders and Huber, 2010; McMurdie and Holmes, 2014). “Differential abundance” refers to different sample means for an individual OTU in contrasting treatments (ex.  $^{13}\text{C}$  vs  $^{12}\text{C}$ ). To calculate

differential abundance, we defined three “heavy” fractions windows (fractions of a density indicative of  $^{13}\text{C}$  incorporation): 1.70 – 1.73 g/mL, 1.72 – 1.75 g/mL, and 1.74 – 1.77 g/mL. Three windows were used to account for differences in gradient position of DNA with differing GC-content (based on Youngblut, et al., in prep). DESeq2 was used to calculate the moderated  $\log_2$ -fold change of  $^{13}\text{C}$  labeled:  $^{12}\text{C}$  control proportion mean ratios and corresponding standard errors for each density window (Anders and Huber, 2010). P-values were corrected for multiple comparison with the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). OTUs from the dataset were defined as incorporators if they had an adjusted p-value less than the false discovery rate of 0.10 (Pepe-Ranney et al., 2016).

As a form of independent filtering (Bourgon et al., 2010; Love et al., 2014), sparsity filters were applied to the dataset. A sparsity filter removes OTUs with sequence counts that fail to reach a user defined sparsity threshold. We tested sparsity thresholds from 0.05 – 0.95 in steps of 0.05. For each density window, substrate, and day combination, the sparsity filter that maximized incorporator number was chosen (Supplemental Figure 4.2) (Pepe-Ranney et al., 2016, Youngblut et al., in prep).

#### *Hierarchical Clustering of Log<sub>2</sub>-Fold Change Response*

To identify response groups of OTUs for each substrate, we used the “cutreeHybrid” function from the R package “dynamicTreeCut” to hierarchically cluster  $\log_2$ -fold change values across tillage and time (Langfelder et al., 2008). We used a average linkage method for hierarchical clustering and deep split value of 0 and a minimum cluster size of 20 OTUs as parameters in “cutreeHybrid”.

## 4.4 Results

### 4.4.1 Soil Biogeochemistry

Tillage significantly decreased unfractionated *in situ* soil C, soil N, and increased soil C:N (Table 4.1, soil C : F-value = 77.2,  $p < 0.001$ ; soil N : F-value = 127.9,  $p < 0.001$ ; C:N : F-value = 14.7,  $p = 0.004$ ), compared to untilled soils. However, for the SIP experiment, till and no-till soils received the same amount of carbon substrate. After adding substrate, overall soil respiration rates differed significantly between no-till and till soils (paired t-test,  $p = 0.02$ ). No-till soils had higher production of CO<sub>2</sub> ( $13.9 \pm 1.0$  mg) than tilled soils ( $11.6 \pm 1.5$  mg). Soil temperature, moisture, or pH between treatment plots did not differ between till and no-till plots (Supplementary Figure 4.3, Table 4.1).

**Table 4.1.** Soil characteristics of the long term tillage experiment in Chazy, NY. All measurements, except moisture, were taken from soil in September 2014. Moisture was averaged over the 11 sampling timepoints from July 2014 – November 2015. Differing superscript letters in each column indicate significant differences between treatments

Tillage	%C	%N	C:N	pH	% Moisture
No-till	$2.13 \pm 0.39^a$	$0.18 \pm 0.04^a$	$11.58 \pm 0.32^a$	$6.89 \pm 0.93$	$16.5 \pm 2.47$
Till	$1.50 \pm 0.26^b$	$0.10 \pm 0.02^b$	$14.36 \pm 1.12^b$	$7.74 \pm 0.05$	$16.5 \pm 6.59$

#### **4.4.2 Unfractionated Soil Bacterial Community affected by Tillage and Time**

We sequenced the *in situ* soil used for stable isotope probing and found that tillage has an effect on the variation of the bacterial community (PERMANOVA,  $p = 0.01$ ,  $F\text{-value} = 3.1$ ,  $R^2 = 0.19$ ). No significant differences are seen in richness across tillage, as measured by Faith's PD (t-test,  $p > 0.05$ ; till:  $175 \pm 19$ , no-till:  $160 \pm 20$ ), or evenness, as measured by Pielou's Evenness measure (t-test,  $p > 0.05$ , till:  $0.89 \pm 0.01$ , no-till:  $0.88 \pm 0.01$ ), or a combination of the two, as measured by Shannon index (t-test,  $p > 0.05$ , till:  $7.16 \pm 0.12$ , no-till:  $7.01 \pm 0.17$ ).

We also calculated the coefficient of variation (CV) for each OTU (standard deviation of relative abundance divided by mean of abundance) across tillage to assess whether tillage affects heterogeneity of OTU abundance, with increased CV suggesting increased heterogeneity of OTU abundance across plots. OTUs in no-till samples had significantly greater CVs than OTUs from tilled samples (t-test,  $p < 0.001$ ; no-till :  $1.24 \pm 0.52$ , till :  $1.09 \pm 0.54$ ). Till samples had a higher skewness of the CV distributions (0.46) as compared to no-till samples (0.14).

We also sequenced the unfractionated soil for each time point, land management, and substrate treatment in the experiment. Both tillage and sampling date have an effect on the variation of the bacterial community (Supplemental Figure 4.4, PERMANOVA, tillage:  $p = 0.001$ ,  $F\text{-value} = 19.9$ ,  $R^2 = 0.14$ ; time:  $p = 0.001$ ,  $F\text{-value} = 20.8$ ,  $R^2 = 0.15$ . Additionally, there is a small, but significant day and substrate treatment interaction (PERMANOVA:  $p = 0.008$ ,  $F\text{-value} = 2.02$ ,  $R^2 = 0.03$ ). Samples, regardless of tillage status, replicate, or substrate treatment follow similar temporal



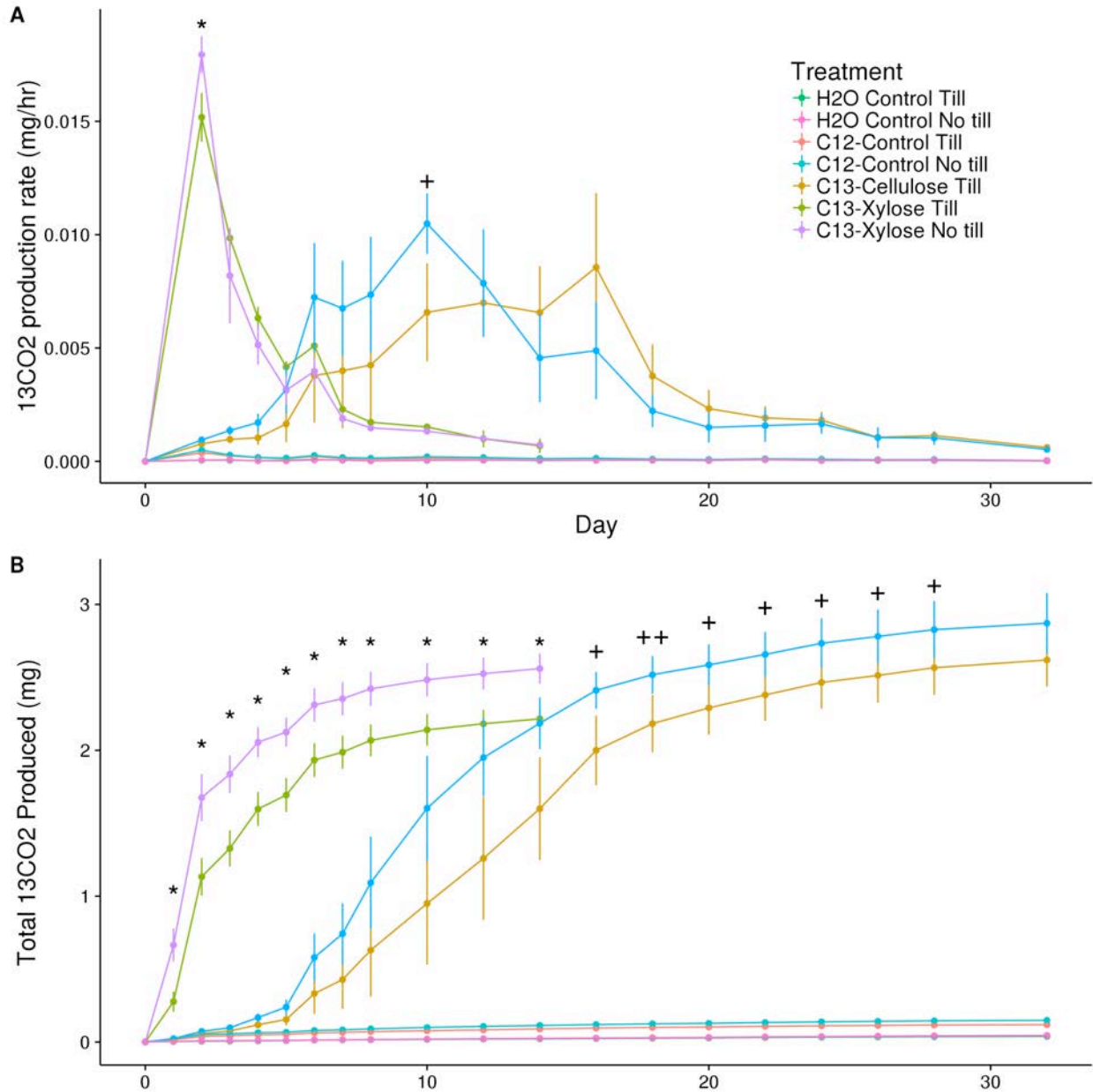
patterns, spreading over NMDS1. When samples are separated into till vs no-till land management, the impact of time is found to be greater for samples under till management (PERMANOVA:  $p = 0.001$ ,  $F\text{-value} = 20.4$ ,  $R^2 = 0.29$ ) than samples under no-till management (PERMANOVA:  $p = 0.001$ ,  $F\text{-value} = 5.7$ ,  $R^2 = 0.11$ ). No-till samples show greater variation over replicate block as compared to till samples (PERMANOVA results)

#### **4.4.3 $^{13}\text{CO}_2$ Production differs between Tillage Conditions for Xylose and Cellulose Substrates**

The addition of water increased soil respiration rate, with no differences in rate across tillage, but did not result in meaningful amounts of  $^{13}\text{C}$   $\text{CO}_2$  released from SOM (0.039 mg and 0.045 mg total  $^{13}\text{C}$  respired from SOM pools during the duration of the experiment for till and no-till, respectively). Measurement of  $^{13}\text{CO}_2$  from soils incubated with  $^{13}\text{C}$ -xylose show rates peaking around day 1 (Figure 4.1a). Significant differences in rate of  $\text{CO}_2$  production occur between no-till and till soils at day 1 during the experiment (paired t-test,  $p = 0.05$  and  $0.02$ , respectively), and these rate differences result in significant differences in cumulative  $^{13}\text{CO}_2$  production from xylose throughout the duration of the experiment (Figure 4.1b). No-till soils release  $2.56 \pm 0.1$  mg of  $^{13}\text{C}$  from  $^{13}\text{CO}_2$  of xylose after 14 days ( $\sim 58\%$  of added  $^{13}\text{C}$ ), compared to  $2.21 \pm 0.08$  mg for tilled soils ( $\sim 50\%$  of added  $^{13}\text{C}$ ) (paired t-test,  $p\text{-value} = 0.03$ ). We find no significant effect of tillage on the amount of  $^{13}\text{C}$  left in soils for xylose samples (paired t-test,  $p = 0.3$ ). However, the mass balance of xylose samples (Supplemental Figure 4.5) indicates

that we are unable to account for all of the carbon in the system (see Supplemental Note 1 for discussion). The mean missing C for samples receiving  $^{13}\text{C}$ -xylose, it is  $0.54 \pm 0.45$  mg (~12%). The mass balance of  $^{13}\text{C}$  recovered in tilled samples is significantly lower from the amount of added  $^{13}\text{C}$  (t-test,  $p = 0.007$ ), while the mass balance of no-till samples is not (t-test,  $p = 0.3$ ).

In the cellulose treatment, no-till soils have a higher rate of  $^{13}\text{CO}_2$  production earlier in the experiment, with rates peaking at day 10 (Figure 4.1a).  $^{13}\text{CO}_2$  production from tilled soils lag, comparatively, and rates peaked around day 16. A significant difference is seen in rates on day 10 (paired t-test,  $p = 0.05$ ). The cumulative production of  $^{13}\text{C}$  from  $^{13}\text{CO}_2$  of cellulose differs between no-till and tilled soils  $2.87 \pm 0.2$  mg vs.  $2.62 \pm 0.18$  mg (~32% and ~29% of added  $^{13}\text{C}$ , respectively), and it is unlikely that this difference results from chance (paired t-test,  $p\text{-value} = 0.06$ , Figure 4.1b). Tillage does not affect the amount of  $^{13}\text{C}$  left in soils between for cellulose samples (paired t-test,  $p = 0.4$ ). However, as with xylose, the mass balance of cellulose samples (Supplemental Figure 4.5) reveals missing carbon. The mean missing C for samples receiving  $^{13}\text{C}$ -cellulose is  $3.2 \pm 1.3$  mg (~36%). The mass balance of both tilled samples and no-till samples is significantly lower than the amount of added  $^{13}\text{C}$  (till: t-test,  $p = 0.004$ , no-till : t-test,  $p = 0.04$ ).

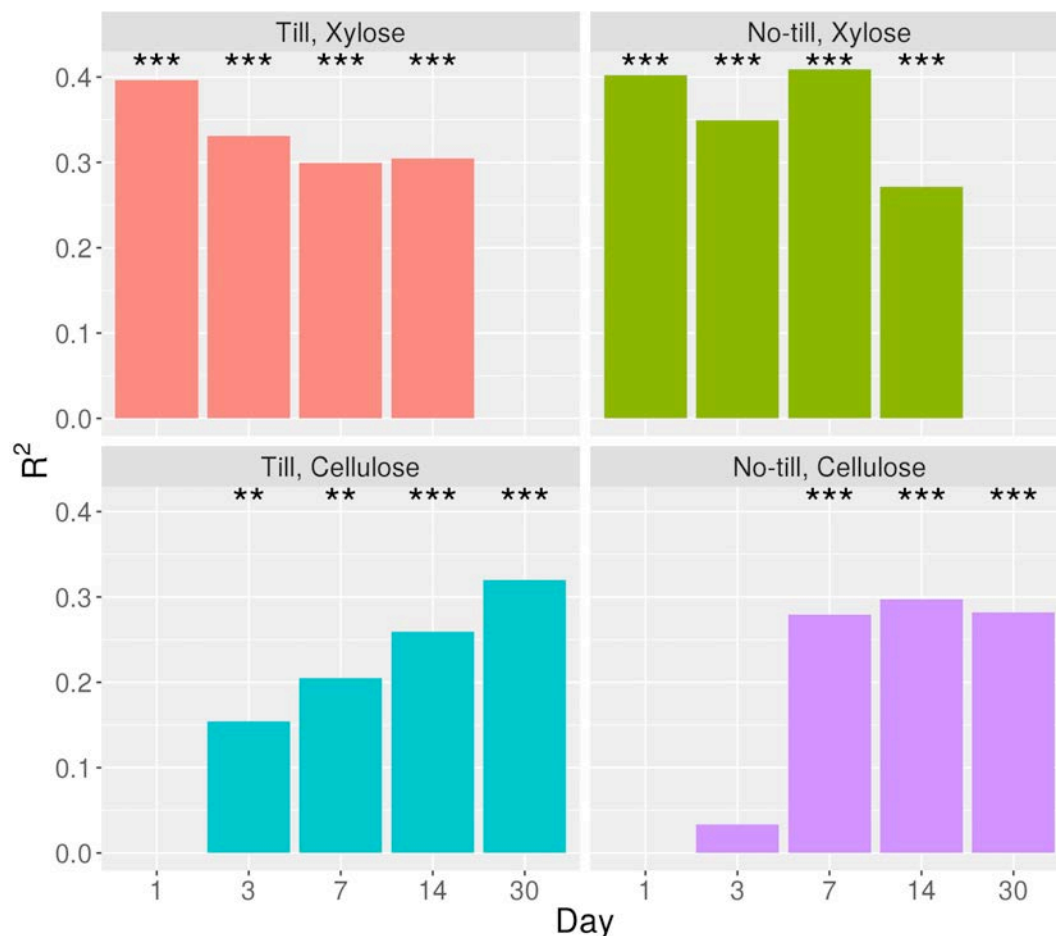


**Figure 4.1.** (A) Mean rates of <sup>13</sup>C production for each microcosm treatment and (B) Mean cumulative <sup>13</sup>C produced throughout the experiment. Colors represent treatment. Stars (\*) represent significant (p ≤ 0.05) differences between xylose till and no-till samples and pluses (+) represent significant differences in cellulose till and no-till samples (paired t-test).

#### **4.4.4 Incorporation of $^{13}\text{C}$ occurs for xylose and cellulose in bacterial communities**

Comparing OTU composition of high-density fractions from  $^{13}\text{C}$  vs.  $^{12}\text{C}$ -substrate treatments provides evidence for bacterial incorporation of  $^{13}\text{C}$  for cellulose and xylose substrates in both no-till and till soils (Figure 4.2, Supplemental figure 4.6). The set of OTUs in the high density fractions receiving corn simulant with  $^{13}\text{C}$ -xylose are significantly different from the set of OTUs in the high density fractions receiving corn simulant with  $^{12}\text{C}$ -xylose on days 1, 3, 7, and 14 for both no-till and till soils (PERMANOVA, Figure 4.2, Supplemental Table 4.1). The percent variation in OTU composition explained as a result of isotopic labeling over the course of the experiment remains consistent for xylose samples, with an average  $R^2$  of  $0.36 \pm 0.06$  for no-till samples and  $0.33 \pm 0.04$  for till samples (Figure 4.2).

Tilled soil communities receiving  $^{13}\text{C}$ -cellulose treatment show evidence of significant isotope incorporation into OTUs on days 3, 7, 14, AND 30 (PERMANOVA, Figure 4.2, Supplemental Table 4.1). No-till soil communities show evidence of significant isotope incorporation into OTUs on days 7, 14, and 30 (PERMANOVA, Figure 4.2, Supplemental Table 4.1). Unlike the  $^{13}\text{C}$ -xylose treatment, the variance attributed to isotope treatment increases over time in the  $^{13}\text{C}$ -cellulose-treated samples, with up to 30% of variance in community composition explained by isotope incorporation by day 30 (Figure 4.2).



**Figure 4.2.**  $R^2$  values representing amount of variance explained by isotopic labeling of DNA in each substrate – tillage management combination over time. A higher  $R^2$  means that more community variation between  $^{12}\text{C}$  and  $^{13}\text{C}$  substrate treatments is explained by incorporation of heavy isotope. Significant community differences due to isotope incorporation, as measured by PERMANOVA, are demarcated by stars ( \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

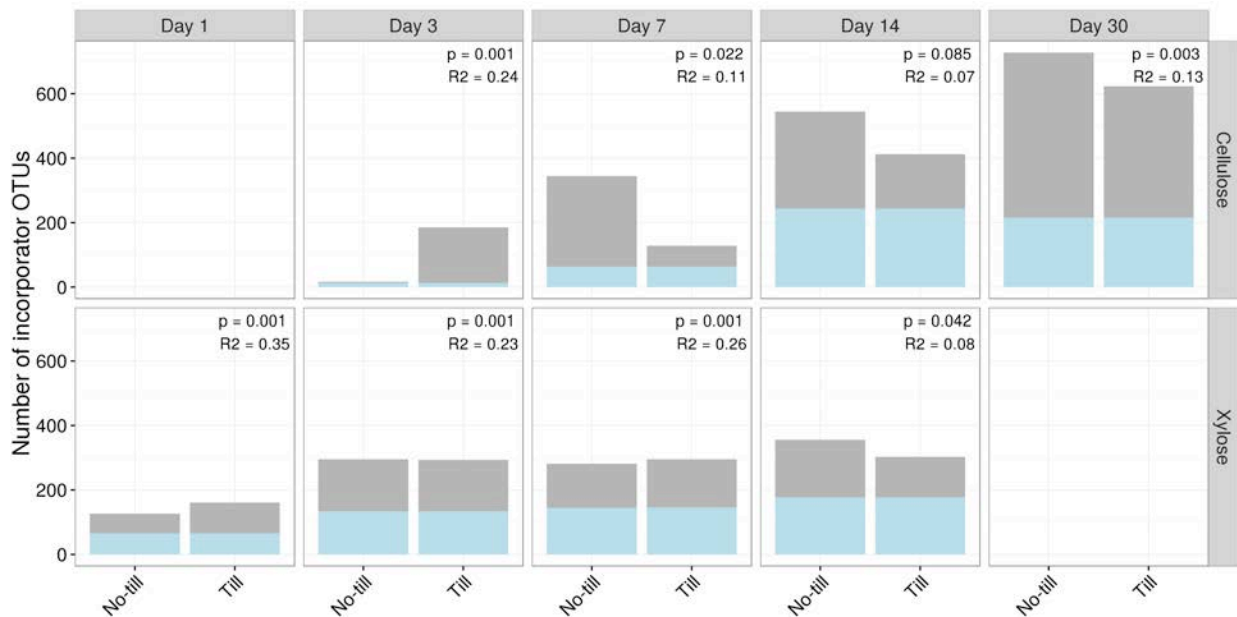
#### 4.4.5 Beta Diversity of OTUs that Incorporate $^{13}\text{C}$ from Cellulose and Xylose

##### *Differs by Tillage*

We also compared composition of xylose incorporator OTUs between till and no-till samples (Figure 4.3). The set of xylose incorporators show significant differences in composition attributed to tillage throughout the experiment (PERMANOVA, Figure 4.3, Supplemental Table 4.2), but the variation attributed to tillage status diminishes over

time from 35% to 8% (Figure 4.3).

The composition of cellulose incorporator OTUs also differs due to tillage for days 3, 7, and 30, but not for day 14 (PERMANOVA, Figure 4.3, Supplemental Table 4.1). The variation due to tillage status also reduces over time, from 24% to 7% from days 3 to 14, but increases on day 30 to 13% (Figure 4.3).



**Figure 4.3.** Number of incorporator OTUs identified for cellulose and xylose substrates over time. Community composition of incorporator OTUs was compared between tillage managements at each time and for each substrate using PERMANOVA. P-values and  $R^2$  values from the tests are shown in each facet, with significant p-values indicating incorporator communities that differ significantly due to tillage and higher  $R^2$  values indicating larger amounts of variation in bacterial community composition explained by tillage. Blue shading indicates number of shared OTUs at each treatment-time combination.

#### 4.4.6 Number of Taxa Incorporating $^{13}\text{C}$ Differ by Land Management, Substrate, and Time

Evidence of  $^{13}\text{C}$  incorporation into specific OTUs is found by comparing the differential abundance of an OTU in corresponding high-density fractions of  $^{12}\text{C}$

substrate treatment and  $^{13}\text{C}$  substrate treatment (DESeq2, Love et al., 2014). OTUs that are significantly enriched in  $^{13}\text{C}$  high-density fractions compared to  $^{12}\text{C}$  high-density fractions are referred to as incorporators (see methods for a user-defined statistical description of incorporator). No-till samples had more incorporator OTUs than till samples for the cellulose treatment, but not for the xylose treatment (Figure 4.3). The number of xylose incorporators is similar over time in till and no-till samples, but the number of cellulose incorporators differs across tillage, especially early in the experiment (Figure 4.3).

604 OTUs are identified as xylose incorporators across time in the till treatment. The number of incorporators in tilled samples increases from day 1 to day 3 and then remains constant through day 14 (Figure 4.3, Day 1: 161, Day 3: 293, Day 7: 296, Day 14: 303). 582 OTUs were identified as xylose incorporators across time in the no-till treatment. No-till incorporators follow a similar temporal pattern to till incorporators, although the number of incorporators increases slightly on day 14 (Figure 4.3, Day 1: 127, Day 3: 295, Day 7: 282, Day 14: 356). Xylose treatments of till and no-till share 352 OTUs. Over time, the number of shared OTUs in the xylose treatment increases (Figure 4.3). OTUs that are not shared between tillage managements of the same substrate treatment are generally incorporators at only one time point (78% of no-till-xylose incorporators, 75% of till-xylose incorporators), while fewer respond at two (18% of no-till-xylose incorporators, 17% of till-xylose incorporators), or three or more time points (4% of no-till-xylose incorporators, 8% of till-xylose incorporators).

In the cellulose treatments, no-till samples have 1,062 incorporator OTUs. The

number of incorporators is low on day 3 but rapidly and continuously increases from day 7 to day 30 (Figure 4.3, Day 3 : 17, Day 7: 345, Day 14: 545, Day 30 : 728). Till samples have 935 incorporator OTUs. There are more incorporators in till than no-till on day 3 (185 OTUs). The number of till incorporators dips on day 7 before rising again, but still lagging no-till incorporator numbers, on day 14 and 30 (Figure 4.3, Day 7: 128, Day 14: 412, Day 30: 623). Cellulose samples share 440 incorporator OTUs across tillage. In the cellulose treatment, the number of shared incorporators increases to a maximum on day 14 and then decreases at day 30, mimicking community composition changes across tillage (Figure 4.3). Like xylose incorporators, the majority of cellulose incorporators that are not shared between tillage respond at one time point (74% of no-till cellulose incorporators, 76% of till cellulose incorporators). There are 20% of no-till and 23% of till unshared cellulose incorporators responding at two time points, with 6% of no-till 0.6% of till unshared cellulose incorporators responding at three time points.

#### **4.4.7 Characterization of Xylose Incorporators**

Significant xylose incorporator OTUs at all four time points fall into several phyla, including *Proteobacteria* (838 OTUs), *Actinobacteria* (528 OTUs), *Bacteroidetes* (220 OTUs), *Planctomycetes* (150 OTUs), *Verrucomicrobia* (137 OTUs), *Firmicutes* (96 OTUs), and *Chloroflexi* (57 OTUs) (Figure 4.4, Supplemental Figure 4.7). Incorporator OTUs classified as members of *Candidate Division BRC1* (17 OTUs) and *Cyanobacteria* (15 OTUs) are found on days 3, 7, and 14, while OTUs from *Candidate Division WS3* (32 OTUs) are found on days 1 and 14. Phyla with incorporators



numbering less than 10 include *Acidobacteria*, *Armatimonadetes*, *Candidate Division TM7*, *Fibrobacteres*, *Gemmatimonadetes*, *Lentisphaerae*, *Spirochaetes*, TA06, and WCHB1-60.

We can compare enriched OTUs from no-till and till soils and identify phyla that have skewed enrichment across land management (ie. greater number of incorporators in one tillage management compared to the other). Significantly higher numbers of xylose incorporators in till soils are found for *Bacteroidetes*, *Chloroflexi*, and *Firmicutes* (Fisher's Exact Test, all  $p < 0.004$ ). *Betaproteobacteria* and *Gammaproteobacteria* have higher number of incorporator OTUs in no-till soils (Fisher's Exact Test, all  $p < 0.001$ ). Number of incorporators are similar between tillage managements for *Alphaproteobacteria*, *Deltaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Candidate Division BRC1*, *Candidate Division TM7*, *Candidate Division WS3*, *Cyanobacteria*, *Fibrobacteres*, *Gemmatimonadetes*, *Lentisphaerae*, *Planctomycetes*, *Spirochaetes*, *Verrucomicrobia* (Supplemental Figure 4.7).

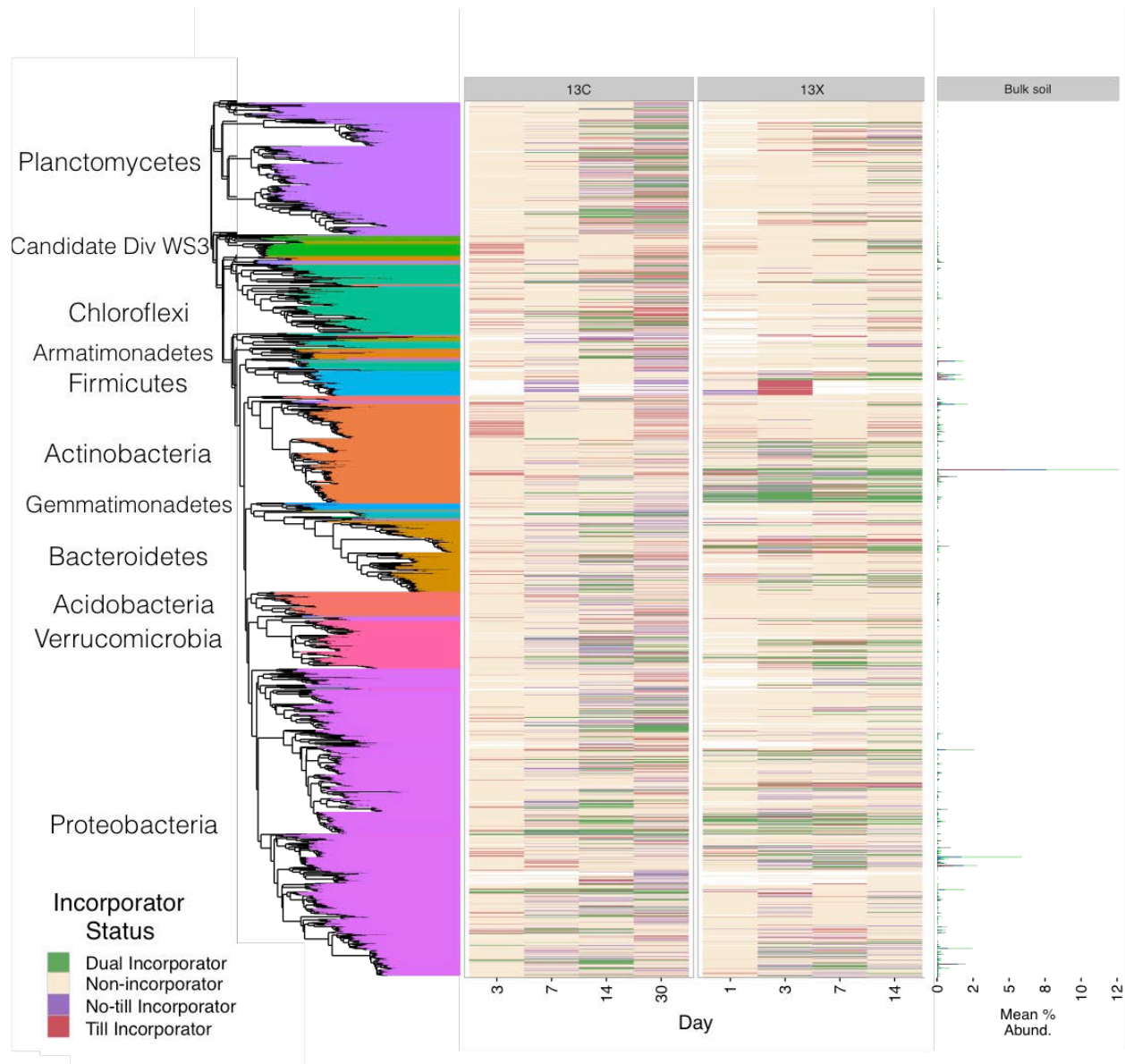
For most phyla, though, numerical dominance of xylose-incorporator OTUs in a certain tillage condition is not temporally consistent (Figure 4.4, Supplemental Figure 4.7). A significantly higher number of till incorporators on day 1 and 7 is seen for *Bacteroidetes* incorporators, with no difference on days 3 and 14 (Fisher's Exact Test, adjusted  $p < 0.02$  for both time points). Till only incorporators are seen for *Candidate Division WS3* on day 1, but similar numbers across tillage is seen by day 14 (Fisher's Exact Test, adjusted  $p = 0.001$ ). Similar levels across tillage from days 1-7, with higher number of till incorporators on day 14 is seen in the *Chloroflexi* incorporators (Fisher's

Exact Test, adjusted  $p = 0.005$ ). A similar number of no-till incorporators on day 1, greater numbers in till on day 3 and then similar numbers across tillage on days 7-14 is characteristic of *Fimicutes* incorporators (Fisher's Exact Test, adjusted  $p < 0.001$ ). Presence in no-till only on day 1, significantly higher numbers in till on day 3 and 7, and then a return to similar numbers across tillage on day 14 is seen for *Planctomycetes* incorporators (Fisher's Exact Test, adjusted  $p = 0.004$  for both time points). Significantly higher numbers of incorporators in no-till than till on days 3 and 14, but not days 7 and 30 is characteristic of *Betaproteobacteria* (Fisher's Exact Test, adjusted  $p < 0.04$  for both) and *Gammaproteobacteria* (Fisher's Exact Test, adjusted  $p < 0.02$  for both time points), while *Deltaproteobacteria* incorporators (Fisher's Exact Test, adjusted  $p = 0.04$ ) differ significantly only on day 7. No significant difference is seen across tillage at any time point for *Alphaproteobacteria*, *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Candidate Division BRC1*, *Cyanobacteria*, *Fibrobacteres*, *Gemmatimonadetes*, *Lentisphaerae*, *Spirochaetes*, and *Verrucomicrobia* (Fisher's Exact Test, adjusted  $p$ -value  $> 0.05$ ).

#### **4.4.8 Xylose Response Groups Differ by Tillage over Time**

We do not see large phylum-level differences between till and no-till xylose incorporators (Figure 4.4). Therefore, we used hierarchical clustering of OTU abundance changes due to isotopic incorporation over time ( $\log_2$ -fold change values) to identify specific response groups. We identified 12 different response groups (Figure 4.5). Groups contained 13 – 37 distinct bacterial families (mean :  $25 \pm 7$  families) and have between 23 – 117 incorporators (mean :  $70 \pm 32$  incorporators). When each

response group is subset to the top 5 families, in terms of number of incorporators, 83 - 100% of group incorporators are represented (mean: 94%  $\pm$  0.6%).

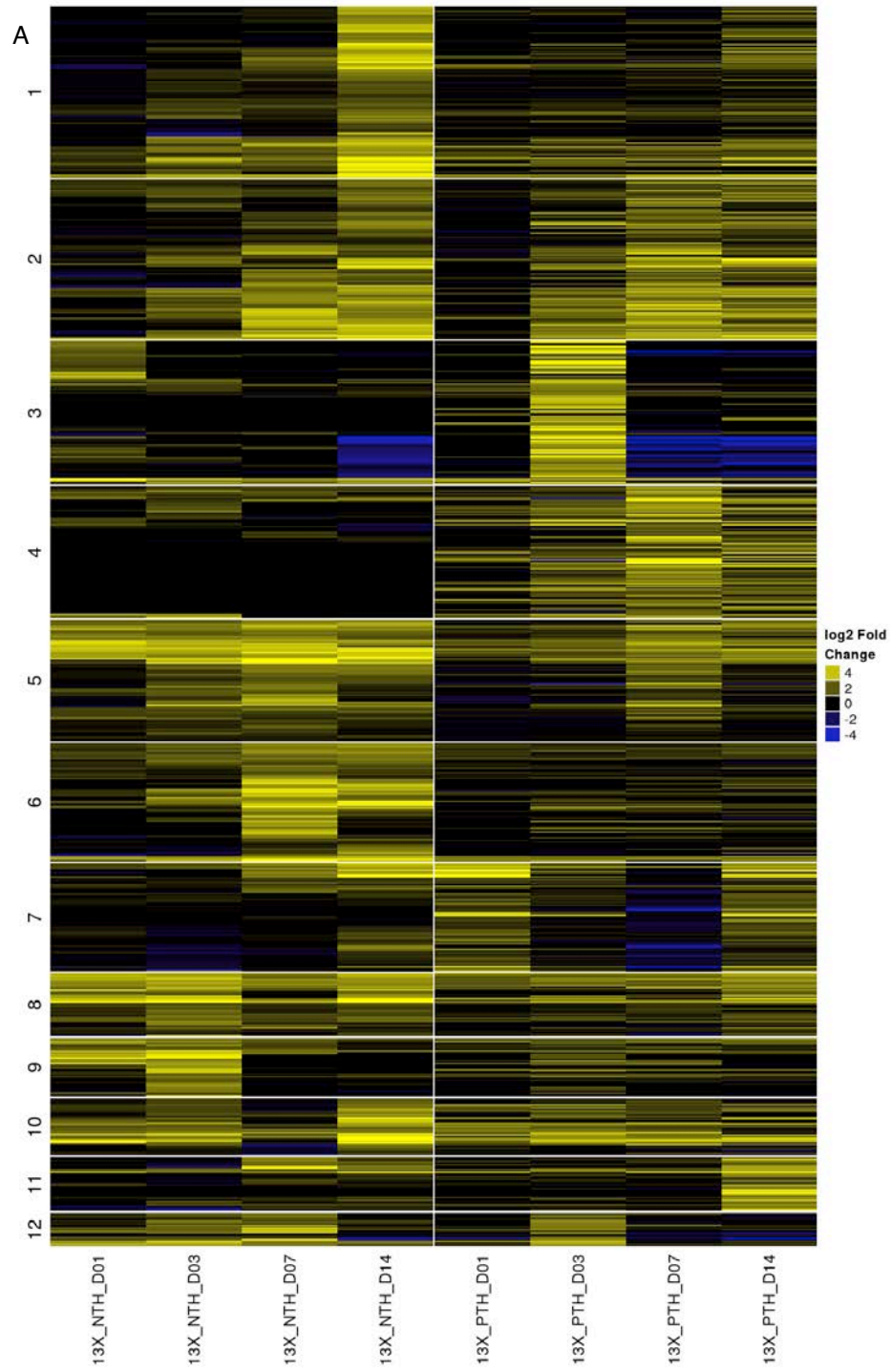


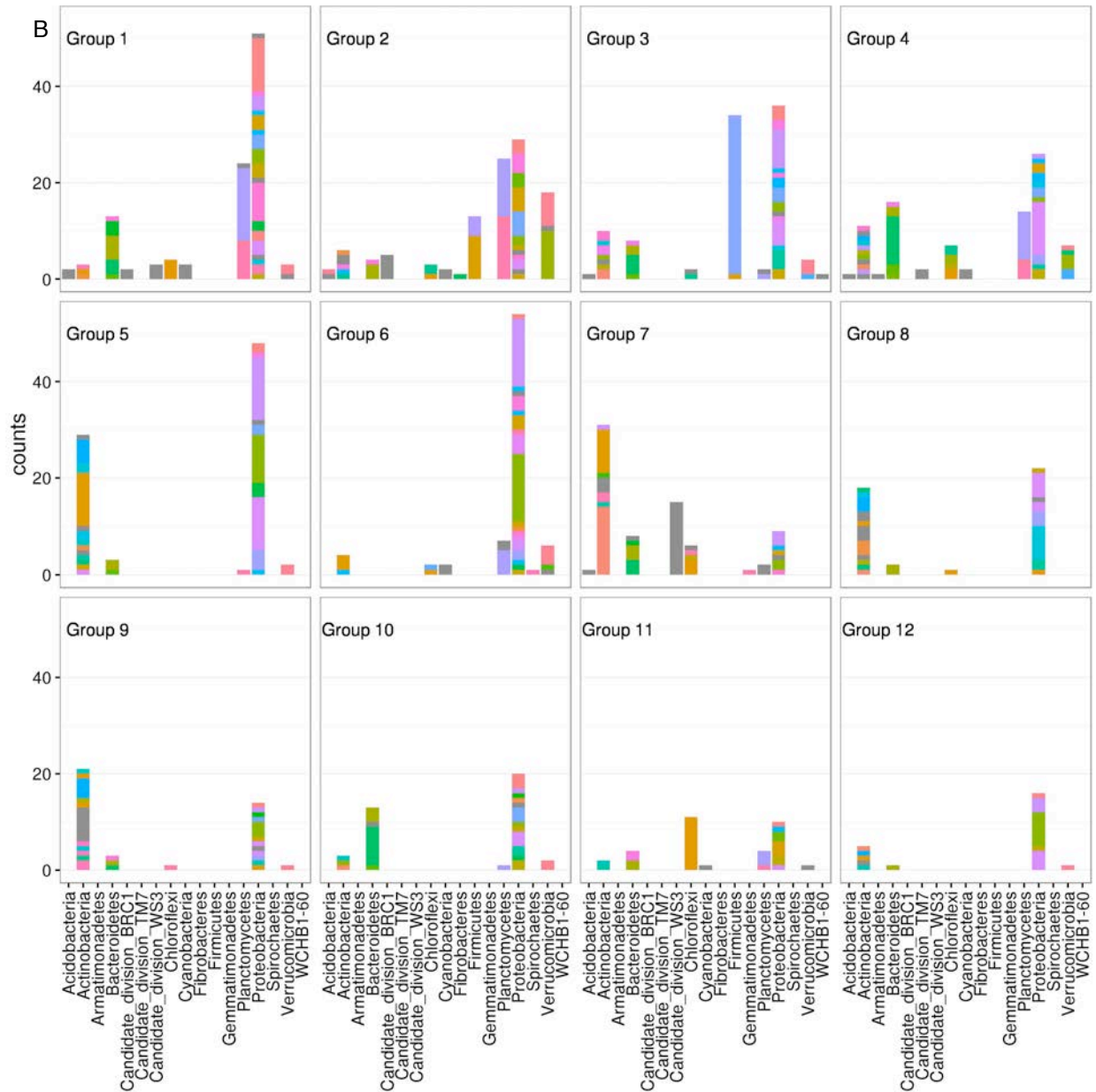
**Figure 4.4.** Phylogenetic tree of incorporator OTUs. Incorporation status by tillage over time is shown on heatmaps for  $^{13}\text{C}$ -cellulose (left heatmap) or  $^{13}\text{C}$ -xylose (right heatmap). The third panel shows percent relative abundance for each OTU in unfractionated soil.

Some groups (Group 1, 2, 10, and 11) contain clusters of OTUs with similar patterns of enrichment across tillage and time. However, some groups differ by tillage

and time. Specifically, on day 1 and 3, groups 5, 8, and 9 contain OTUs that are more highly enriched in the  $^{13}\text{C}$ -xylose no-till treatment than the  $^{13}\text{C}$  xylose till treatment. These groups are dominated by OTUs from *Actinobacteria* and *Proteobacteria*. Incorporators in these groups come from 58 different families, with the majority from *Arthrobacter*, *Norcardioides*, unknown *Microbacteriaciaceae*, *Agromyces*, *Rhodobacteraceae*, *Pseudomonadaceae*, *Comamonadaceae*, *Phyllobacteriaceae*, and *Methylobacteriaceae*. By day 7, no-till specific incorporators mainly are seen in groups 5 and 6. Again, these groups are dominated by OTUs from *Actinobacteria* and *Proteobacteria*. Incorporators come from 48 families, with the majority from *Arthrobacter*, *Pseudomonadaceae*, *Comamonadaceae*, *Rhodobacteraceae*, and *Phyllobacteriaceae*.

OTUs more highly enriched in till than no-till on day 1 are found in group 7, containing a diversity of phyla (*Candidate Division WS3*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Planctomycetes*, and *Proteobacteria*). However, within these phyla only a 26 families account for the incorporators, with the majority coming from *Acidimicrobiaceae*, *Arthrobacter*, and an “uncultured bacterium” of *Candidate Division WS3*. On day 3, group 3 consists of more highly enriched incorporator OTUs in till than no-till. This group contains 39 families and is dominated by *Firmicutes* (*Paenibacilliaciaceae*), and *Proteobacteria* (*Pseudomonadaceae*, *Hyphomicrobiaceae*, *Xanthomonadaceae*, *Oxalobacteraceae*).





**Figure 4.5. (A)** Functional groups (numbered) of xylose incorporator OTUs clustered by log<sub>2</sub>-fold change value of isotope incorporation. Color indicates log<sub>2</sub>-fold change in abundance in the <sup>13</sup>C treatment vs <sup>12</sup>C treatment (yellow indicates higher abundance in <sup>13</sup>C, while blue indicates higher abundance in <sup>12</sup>C). Columns represent log<sub>2</sub>-fold change values in each land management (no-till in the left four columns, till in the right four columns) at each time point. **(B)** Taxonomic composition of functional groups. OTUs from each group are arranged by phylum on the x-axis with numbers of OTUs on the y-axis. OTUs within each phylum are colored by family annotation.

By day 7, group 4 contains incorporator OTUs more highly enriched incorporator OTUs in till than no-till. This group contains several phyla (*Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Proteobacteria*, and *Verrucomicrobia*) and 42 families, with the majority of incorporators from *Flavobacteriaceae*, *Planctomycetaceae*, and *Rhodobactereaceae*.

By day 14, differences between till and no-till incorporator response groups become less, with the exception of group 4 (incorporators from *Rhodobacteraceae*, *Flavobacteraceae*, and *Planctomycetaceae*), which shows till-specific response over the course of the experiment. These response groups indicate that distinct groups of incorporators exist for till and no-till samples at specific time points over the experiment. The OTUs within these groups are not conserved at the phylum level. However, a small number of families (~5) make up the majority of the incorporator pool of each group and these families differ over time between till and no-till, suggesting that there is a phylogenetic specificity to groups involved in xylose cycling in no-till vs till soils.

#### **4.4.9 Characterization of Cellulose Incorporators**

Phyla with large numbers of significant incorporators of  $^{13}\text{C}$  from  $^{13}\text{C}$ -cellulose at all four time points include *Proteobacteria* (1125 OTUs), *Planctomycetes* (513 OTUs), *Chloroflexi* (277 OTUs), *Bacteroides* (251 OTUs), *Actinobacteria* (241 OTUs), *Verrucomicrobia* (212 OTUs), *Acidobacteria* (89 OTUs), *Cyanobacteria* (26 OTUs), *Nitrospirae* (21 OTUs), and *Gemmatimonadetes* (17 OTUs) (Figure 4.4, Supplemental Figure 4.8). The phyla *Armatimonadetes* (46 OTUs), *Fibrobacteres* (39 OTUs),

*Candidate Division WS3* (37 OTUs), *Firmicutes* (37 OTUs), and *Spirochaetes* (16 OTUs) also contain several significant incorporator OTUs, but not at all four time points. Responder OTUs classified as *Armatimonadetes* and *Fibrobacteres* are found on days 7, 14, and 30. Phyla containing responder OTUs at only two time points include *Candidate Division WS3* (Day 3 and 30), *Firmicutes* (Day 7 and 30), and *Spirochaetes* (Day 14 and 30). Other phyla, including *BD1-5*, *Candidate Division BRC1*, *Candidate Division OD1*, *Candidate Division OP3*, *Candidate Division TM7*, *Chlamydiae*, *Chlorobi*, *Elusimicrobia*, *JL-ETNP-Z39*, *TA-06*, and *TM6* each contained less than 10 incorporator OTUs.

Significantly greater number of OTU responders in till management is seen for *Actinobacteria*, and *Chloroflexi*, (Fisher's Exact Test, all adjusted p-values < 0.004), while greater numbers in no-till management is seen for *Bacteroidetes*, *Candidate Division WS3*, *Firmicutes*, *Planctomycetes*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* (Fisher's Exact Test, all adjusted p-values < 0.01). A similar number of incorporators across tillage is seen for *Alphaproteobacteria*, *Acidobacteria*, *Armatimonadetes*, *Candidate Division BRC1*, *Cyanobacteria*, *Gemmatimonadetes*, *Spirochaetes*, *Nitrospirae*, and *Planctomycetes* (Fisher's Exact Test, all adjusted p-values > 0.05).

However, difference in incorporator number between tillage is not static over time (Figure 4.4, Supplemental Figure 4.8). More incorporators are seen in till compared to no-till on day 3 (Fisher's Exact Test, all adjusted p-values < 0.002). Of these phyla, nearly all have significantly higher incorporator numbers in no-till as compared to till



later on in the experiment: *Actinobacteria* (Day 7,  $p = 0.03$ ), *Bacteroidetes* (Day 7,  $p < 0.001$ ), *Alphaproteobacteria* (Day 7,  $p < 0.001$ ), *Deltaproteobacteria* (Day 14,  $p = 0.001$ ), and *Gammaproteobacteria* (Day 30,  $p < 0.001$ ). Consistently higher number of incorporators in tilled soils is only seen in *Candidate Division* WS3 (Day 30,  $p < 0.001$ ). An increased number of incorporators in no-till compared to till on day 30, but comparable numbers at other time points, is seen for *Firmicutes* and *Gemmatimonadetes* incorporators ( $p < 0.001$  and  $p = 0.03$ , respectively). A higher number of no-till incorporators on day 7 is characteristic of *Planctomycetes* ( $p = 0.04$ ) and *Verrucomicrobia* incorporators ( $p < 0.001$ ).

#### **4.4.10 Cellulose Response Groups Differ by Tillage over Time**

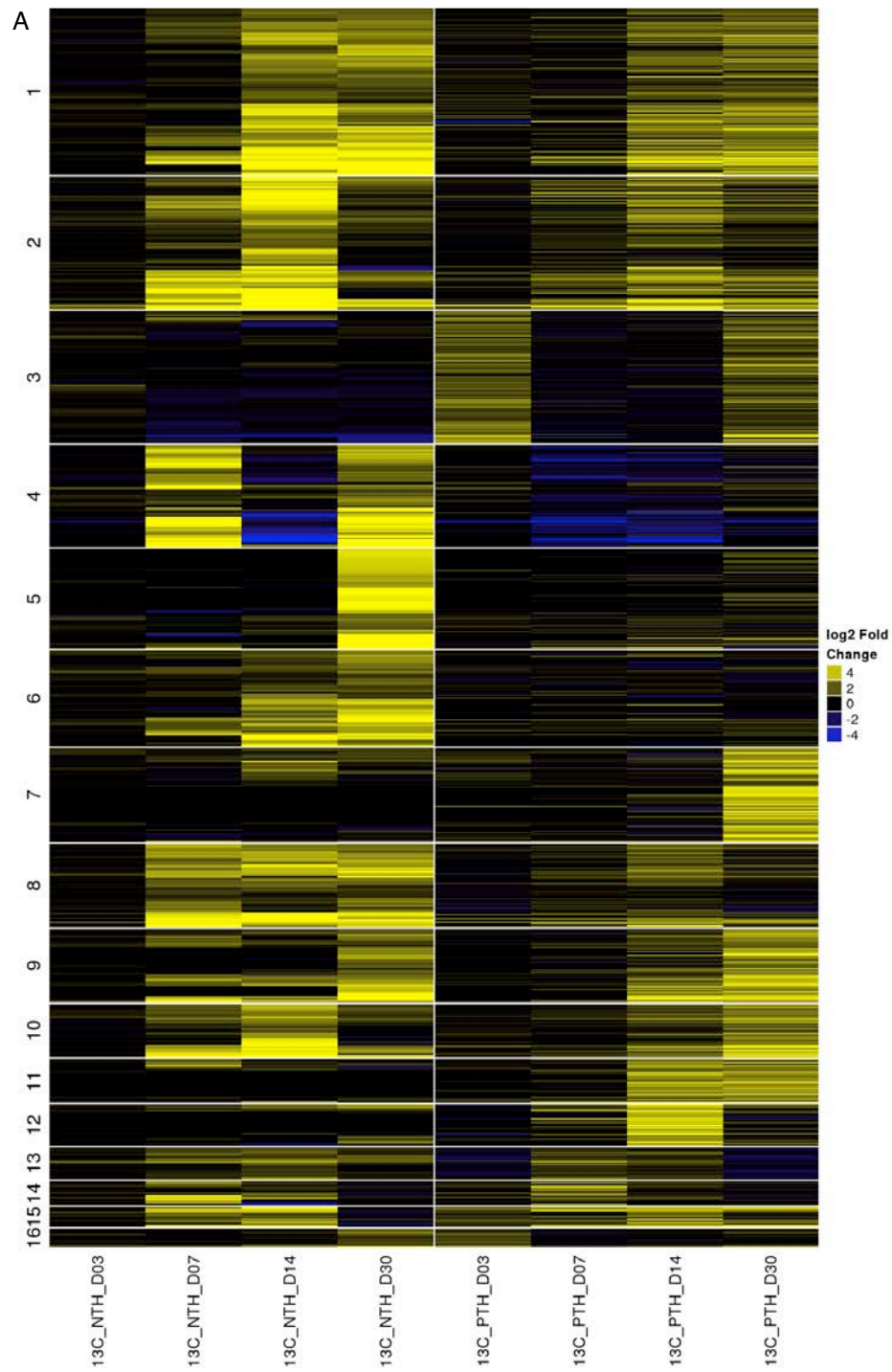
As with xylose, we do not see large phylum-level differences between till and no-till samples over time (Figure 4.4). Again, we looked at hierarchical clustering of  $\log_2$ -fold change values for till and no-till incorporators over time to establish response patterns and examine phylogenetic relationship within response groups.

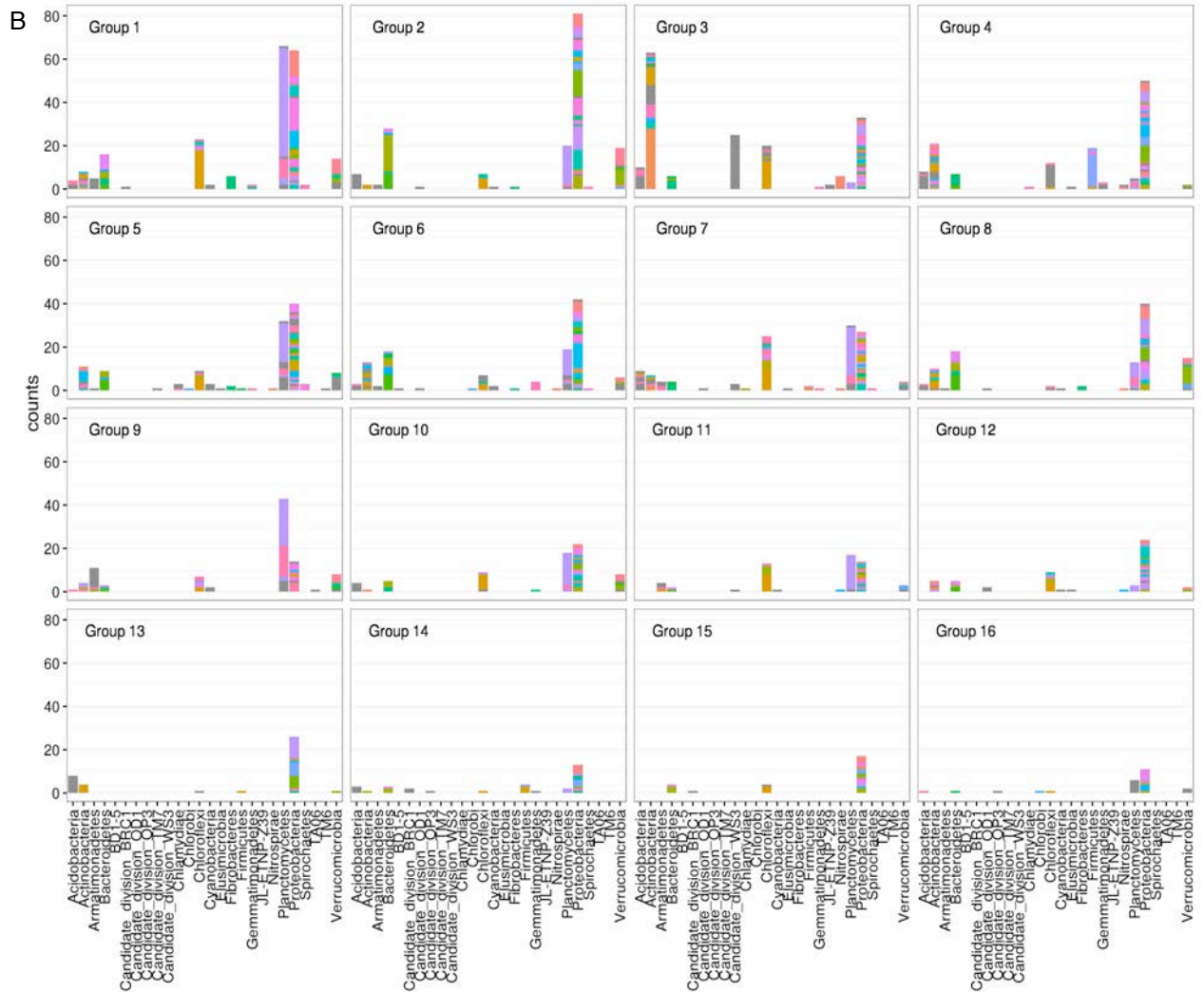
We identified 17 different response groups (Figure 4.6). Groups contained 11 – 50 distinct bacterial families (mean :  $28 \pm 15$ ) and have between 24 – 203 incorporators (mean :  $87 \pm 61$ ). When each response group is subset to the top 5 families, based on number of incorporators, 22 -100% of group incorporators are represented (mean  $48\% \pm 30\%$ ).

Groups 1, 9, 13, 14, 15, 16 showed similar patterns of response between till and no-till samples over time. A greater number of till incorporators exist on day 3 and these

OTUs belong to response group 3, containing 46 families. The majority of these incorporator OTUs belong to the families *Acidomicrobiaceae* (Acidobacteria), an uncultured *Actinobacteria* bacteria, an uncultured *Candidate Division* WS3 bacteria, and *Anaerolineaceae* (Chloroflexi). No response groups show increased log<sub>2</sub>-fold change intensity on day 7 for till incorporators, but by days 14 and 30, groups 11 and 12 do show higher till response than no-till response. Within these groups, there are 52 families. The largest number of incorporators come from the families *Planctomycetaceae* (*Planctomycetes*), *Anaerolineaceae* (*Chloroflexi*), *Chloroflexus* (*Chloroflexi*), *Sphingomonadaceae* (*Proteobacteria*), and *Legionellaceae* (*Proteobacteria*).

By day 7, no-till incorporators show higher log<sub>2</sub>-fold change values as compared to till incorporators, as seen in response groups 2, 4, 8, and 10. These four response groups contain 48 families. The majority of incorporators belong to *Planctomycetaceae* (*Planctomycetes*), *Comomonadaceae* (*Proteobacteria*), *Chitinophagaceae* (*Bacteroidetes*), *Xanthomonadaceae* (*Proteobacteria*), *Chthoniobacteraceae* (*Verrucomicrobia*), *Pseudomonadaceae* (*Proteobacteria*), and *Verrucomicrobiaceae* (*Verrucomicrobia*). By day 14, a similar set of no-till incorporators are seen to show higher log<sub>2</sub>-fold change values, including groups 2, 8, 10, as well as group 6. Group 6 contains 55 families, with a majority of incorporators in *Planctomycetaceae* (*Planctomycetes*), *Nannocystineae* (*Proteobacteria*), and *Cytophagaceae* (*Bacteroidetes*).





**Figure 4.6. (A)** Functional groups (numbered) of cellulose incorporator OTUs clustered by log<sub>2</sub>-fold change value of isotope incorporation. Color indicates log<sub>2</sub>-fold change in abundance in the <sup>13</sup>C treatment vs <sup>12</sup>C treatment (yellow indicates higher abundance in <sup>13</sup>C, while blue indicates higher abundance in <sup>12</sup>C). Columns represent log<sub>2</sub>-fold change values in each land management (no-till in the left four columns, till in the right four columns) at each time point. **(B)** Taxonomic composition of functional groups. OTUs from each group are arranged by phylum on the x-axis with numbers of OTUs on the y-axis. OTUs within each phylum are colored by family annotation.

#### **4.4.11 Alpha Diversity does not Differ Among Incorporator Communities**

We tested both richness and evenness of incorporator OTU communities from each land management and substrate combination in unfractionated *in situ* DNA through calculation of Faith's Phylogenetic Diversity (PD), Shannon index, and Pielou's evenness score. Faith's PD is a measure of community richness that calculates the sum of the total phylogenetic branch length in a community (Faith, 1992). The Shannon index contains information about both community richness and evenness, as it calculates the proportion of an individual member relative to the total membership of the community, multiplied by the natural logarithm of the proportion. Values are then summed across community members (Shannon, 1948). Pielou's evenness is calculated by dividing the Shannon index by total species richness in a community, and thus allows for the isolation of community evenness (Pielou, 1966).

Communities of cellulose incorporators in till managements have higher mean Faith's PD, Shannon Index, and Pielou's evenness scores than no-till soils, but differences are not significant (paired t-tests,  $p > 0.05$ , Table 4.2). Communities of xylose incorporators in till and no-till managements also have no significant difference in the Shannon index and Pielou's evenness, or Faith's PD than no till soils (paired t-test,  $p > 0.05$ , Table 4.2). Xylose samples also show lower Faith's PD and Shannon Index than communities of cellulose incorporators. We also tested the richness and evenness of the entire no-till and till communities, without subsetting to incorporators, and found no significant differences between the three metrics in the two tillage managements (paired t-test,  $p > 0.05$ , Table 4.2).

**Table 4.2.** Alpha Diversity metrics calculated for incorporator communities of each substrate - tillage management combination and the entire community of each tillage management. Values of each metric were compared within substrate, across tillage management (paired t-test) and no significant differences were found at level of  $p < 0.05$

Community	Faith's PD	Shannon Index	Pielou's Evenness
Cellulose - Till Incorporators	51.4 $\pm$ 4.2	5.3 $\pm$ 0.1	0.85 $\pm$ 0.02
Cellulose - No-till Incorporators	47.6 $\pm$ 4.6	5.1 $\pm$ 0.3	0.83 $\pm$ 0.04
Xylose - Till Incorporators	30.3 $\pm$ 2.1	4.7 $\pm$ 0.1	0.82 $\pm$ 0.02
Xylose - No-till Incorporators	26.4 $\pm$ 3.0	4.8 $\pm$ 0.7	0.84 $\pm$ 0.01
No-till, full community	222.4 $\pm$ 9.0	7.2 $\pm$ 0.04	0.87 $\pm$ 0.01
Till, full community	224.5 $\pm$ 30.6	7.4 $\pm$ 0.08	0.89 $\pm$ 0.01

#### **4.4.12 Replicate Microcosms Indicate Biological Heterogeneity in Responder**

##### **OTUs**

We analyzed two additional sets of replicate microcosms for both the  $^{13}\text{C}$ -xylose, day 3 and the  $^{13}\text{C}$ -cellulose, day 30 samples. Replicates, in this case, are biological replicates, with soils taken from two other blocks of the field site. In terms of microcosm incubation and substrate addition, they have been treated identically.

Of the three microcosms tested for the xylose, day 3 treatment, a total 1,050 OTUs identify as significantly enriched in the high density fractions of the  $^{13}\text{C}$  vs the  $^{12}\text{C}$  isotope treatment (Supplemental Figure 4.9). The number of incorporators in no-till soils ranges from 188 - 634 (mean : 369  $\pm$  235), while in till soils numbers range from 152 –

182 (mean :  $165 \pm 15$ ). When compared in a pairwise manner, no-till replicates share between 16 – 95 (1% - 11%) incorporators (mean :  $50 \pm 40$ ), while till responders share between 67 – 84 (27 – 32%) incorporators (mean :  $74 \pm 9$ ).

The replicates tested for the cellulose, day 30 treatment identify 2,230 unique OTUs as significant incorporators (Supplemental Figure 4.10). In no-till soils, the number of incorporators spans 430 – 858 OTUs (mean :  $605 \pm 224$ ) , while in tilled soils it spans 681 – 843 OTUs (mean :  $749 \pm 84$ ). Replicates from no-till soils share between 12 – 300 (1 – 28%) incorporators (mean :  $108 \pm 166$ ), while till soils share between 380 – 404 (32 – 39%) incorporators (mean :  $392 \pm 12$ ). All three replicates share 370 OTUs (17%), but when partitioned out by replicate and tillage management, only 2 OTUs (0.09%) are shared across the group. When looking at unfractionated soil bacterial communities, the no-till communities show greater heterogeneity than till communities (Supplemental Figure 4.4), consistent with the trend in shared incorporators in till and no-till.

## 4.5 Discussion

We performed high-resolution stable isotope (HR-SIP) probing on soils under till and no-till management with cellulose and xylose substrates. Our study confirmed activity of specific taxa in cellulose and xylose degradation processes and identified novel taxa involved. *Proteobacteria* (*Cellvibrio*, *Deviosa*, *Rhizobium*, and *Sorangineae*), *Planctomycetes*, *Verrucomicrobia* (*Spartobacteria*), *Chloroflexi* (*Herpetesiphonales*), *Bacteroidetes* (*Cytophagia*), and *Actinobacteria* were identified as potential cellulose

degraders or cellulose by-product degraders in this study and previous SIP studies (Eichorst and Kuske, 2012; Pepe-Rannek et al., 2016; Schellenberger et al., 2010; Verastegui et al., 2014; Wang et al., 2015). Similar xylose incorporator phyla were identified between our study and those previous, including *Bacteroidetes* (*Flavobacteriia*, *Sphingobacteriia*, and *Cytophagia*), *Firmicutes* (*Bacilli*: both *Bacillaceae* and *Paenibacillaceae*), *Actinobacteria* (mostly *Micrococcales*), and *Proteobacteria* (*Rhizobiales*, *Burkholderiales*, and *Pseudomonadales*) (Pepe-Rannek et al., 2016; Verastegui et al., 2014). However, we also identified a wide variety of other taxa that may participate in cellulose cycling, both within the above phyla and in other phyla not previously observed (a list of all taxa identified as incorporators can be found through the Github link: <https://github.com/chvtnk/ChazySIP>).

We also characterized bacterial community in response to tillage in *in situ* soils from this field site in a previous study (Chapter 2). In the soils at the time point used for this experiment, we found that *Actinobacteria* and *Verrucomicrobia* have higher numbers of OTUs enriched under no-till as compared to tilled conditions. Conversely, *Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, and *Proteobacteria* have higher number of OTUs enriched under till as compared to no-till conditions. Xylose incorporators from phyla *Bacteroidetes* and *Chloroflexi* and cellulose incorporators from *Chloroflexi* followed *in situ* patterns, with higher numbers in till than no-till. However, these phylum-level differences in tillage preference did not always translate to phylum-level differences in incorporators between tillage managements. Certain phyla, such as *Actinobacteria* had greater numbers of cellulose incorporators in



till soils on day 3, opposite to their increased relative abundance in no-till vs tilled soils in *in situ* soils. The same is true for cellulose incorporators from *Bacteroidetes* and *Proteobacteria* that had greater number of cellulose incorporators in no-till than in till. Thus, differences in abundance of specific phyla before the beginning of the incubation do not necessarily predict the dynamics of carbon acquisition seen during the experiment.

We also found differences in  $^{13}\text{CO}_2$  production and communities involved in  $^{13}\text{C}$ -cellulose and  $^{13}\text{C}$ -xylose cycling between tillage managements. Till and no-till soils show differences in  $^{13}\text{CO}_2$  production, with no-till soils releasing larger amounts of  $^{13}\text{CO}_2$  as compared to tilled soils. Consistent with our findings, no-till soils have been shown to have higher beta-glucosidase exo-enzyme activity as compared to tilled soils (Mbutia et al., 2015). Additionally, undisturbed soils, including pasture and no-till soils, show higher rates of mineralization and immobilization of C than tilled soils (Attard et al., 2016; Mbutia et al., 2015). We also saw differences in the microbial communities involved in uptake and metabolism of both  $^{13}\text{C}$ -xylose and  $^{13}\text{C}$ -cellulose. Thus, the microbial communities involved in cellulose and xylose decomposition in till and no-till soils differ and may contribute to functional variation in the decomposition of both substrates.

Controls on the function of microbial communities in soil are debated in the literature. Two competing, but not mutually exclusive, hypotheses include control by abiotic factors and influence of community composition. In the mineral soil environment, abiotic variables such as temperature (Davidson and Janssens, 2006; Davidson et al.,

2000), moisture (Blazewicz et al., 2013; Fierer and Schimel, 2002; Huxman et al., 2004), pH (Fernández-Calviño and Bååth, 2010; Rousk et al., 2009), soil chemistry and structure (Schimel and Schaeffer, 2012; Sollins et al., 1996; Tisdall and Oades, 1982), substrate quality (Cleveland et al., 2013; Fanin et al., 2015) have been implicated in functional changes in carbon cycling. For example, a study looking at the influence of litter quality and community composition on carbon cycling in soil found that two-thirds of variance in carbon fluxes could be explained by litter quality alone (Cleveland et al., 2013).

However, the Cleveland et al. study also found that ~30% of variance in respiration rates could be explained by bacterial community composition. Thus, there is also evidence for the influence of community structure on soil carbon cycling (Kaiser et al., 2014; Placella et al., 2012; Powell et al., 2015; Strickland et al., 2009), nitrogen fixation (Hsu and Buckley, 2008), nitrification (Bouskill et al., 2012), and methane oxidation (Levine et al., 2011). A recent meta-analysis, looking at soil carbon and nitrogen cycling processes using 82 global datasets over a variety of soil types and managements, found that using solely environmental parameters to predict process rates left an average of 44% of variation unexplained (Graham et al., 2016). More variation could be explained when community diversity statistics or functional gene information were included in models. Additionally, the impact of microbial predictors on models in agricultural soil was higher (average adjusted  $R^2 = 0.38$ ) than in other soil systems.

Our experimental set-up helped to control for many abiotic factors, such as pH,

moisture, temperature, soil structure (through sieving of soils), temporal variability, and substrate quality. Significant differences did exist among soils used, including differences in SOM and C:N ratio of that SOM. However, we added the same amount of  $^{13}\text{C}$  to each microcosm, with the same C:N ratio, and focused our measurements on  $^{13}\text{CO}_2$  production and  $^{13}\text{C}$  incorporation into biomass. Thus, we minimized the impact of abiotic variables as much as is experimentally possible.

We cannot negate the fact that the physiological states of these community members may differ between land managements through influence of abiotic factors. Physiology, as impacted by abiotic variables, has been well implicated in ecosystem-level dynamics (Goldfarb et al., 2011; Lee and Schmidt, 2014; Manzoni et al., 2012; Schimel and Weintraub, 2003; Schimel et al., 2007; Sinsabaugh et al., 2013). However, we see differences in community function corresponding with differences in community structure even with control over abiotic variables. Thus, we suggest that variation in composition of bacterial communities across tillage in this system may, in combination with abiotic factors and physiological differences, contribute to variation in function.

The significant differences in community composition in no-till and till soils for both cellulose and xylose cycling at all time points of our experiment indicate support for differences in  $^{13}\text{C}$  incorporator community composition contributing to differences in community function across tillage. On day 3, cellulose incorporators from *Actinobacteria* (*Acidimicrobiaciae* and unknown families), *Bacteroidetes*, *Candidate Division WS3* (unknown families), *Chloroflexi* (*Anaerolineaceae*), *Alphaproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* are enriched in till soils, while there are

relatively few incorporators in no-till soils on Day 3. Tilled soils, being low in C, N, and other nutrients, may select for organisms that quickly respond to nutrient influx, suggesting that environmental variables and community composition and function are linked (Strickland et al., 2009). *Actinobacteria* and *Deltaproteobacteria* show evidence of rapid response in soils under severe drought, able to ramp up protein production and cell growth quickly (1 hour) after water addition (Placella et al., 2012). Rapid cell growth may correspond to higher carbon use efficiency (CUE) and less production of CO<sub>2</sub> (Manzoni et al., 2012; Sinsabaugh et al., 2013). No-till soils, having higher background levels of C, N and other nutrients, may contain a community of organisms that are slower to grow and divide in response to added nutrients, have a lower CUE and, thus, produce more CO<sub>2</sub>. Consistent with these hypotheses, soils under intensive soybean monoculture cropping have higher carbon use efficiencies compared to forest soils (Lee and Schmidt, 2014).

Similarly, in the samples receiving <sup>13</sup>C-xylose, there is a similar trend of greater numbers of incorporators from tilled soils at the beginning of the experiment. Specifically, the phyla *Bacteroidetes*, *Candidate Division WS3*, *Planctomycetes*, and *Firmicutes* had significantly higher numbers of incorporators in till than no-till samples at day 1 and day 3. Among these incorporators is a large bloom of the genus *Paenibacillus* (phylum *Firmicutes*). *Paenibacillus* and other members of the *Firmicutes*, and members of the *Bacteroidetes* have been previously implicated in xylose cycling (Pepe-Ranney et al., 2016). Members of the *Firmicutes* are known to be rapidly-growing and quick to resuscitate from conditions of nutrient deprivation or desiccation (Setlow, 2014). Their

prevalence in the till soils may influence the community CUE, resulting in more  $^{13}\text{C}$  incorporated into biomass rather than released as  $^{13}\text{CO}_2$ . Additionally, members of the *Firmicutes* form spores, facilitating the storage of C in the soil (McKenney et al., 2013). Members of *Bacteroidetes* also become labeled early in response to  $^{13}\text{C}$  xylose addition (Pepe-Ranney et al., 2016), suggesting they may also contribute to incorporation of  $^{13}\text{C}$  into biomass.

Differences in community composition between till and no-till soils, varied over time and by substrate treatment. The communities incorporating  $^{13}\text{C}$  from xylose differed greatly at the beginning of the experiment, but became more similar over time. Similarly communities incorporating  $^{13}\text{C}$  from cellulose became more similar from day 3 to day 14, but by day 30 the amount of variation due to tillage increased in the two communities. The growing similarity over time is reflected in the gas data : for both cellulose and xylose treatments,  $^{13}\text{CO}_2$  rates became more similar over the course of the experiment. However, early differences in rates between tillage managements manifested in continued significant differences in  $^{13}\text{CO}_2$  production for both xylose and cellulose treatments throughout much of the experiment. This suggests that differences in the bacterial community between soils can have effects on the system even after the communities shift to become more similar.

These changes in temporal dynamics of labeling also indicate that carbon cycling within our microcosms is a complicated network. We provide multiple carbon sources, only one of which is  $^{13}\text{C}$  labeled. Therefore, although we can detect  $^{13}\text{C}$  labeling in OTUs, some of these OTUs may also be eating unlabeled carbon. Shifts from

metabolism of labeled to unlabeled carbon or vice-versa may be occurring throughout the experiment, resulting in changes in OTU labeling over time. For example, OTUs in xylose response group 3 is highly labeled by day 3, but many of these OTUs were not detected as incorporators by day 7. It is possible that this group of OTUs exhausted the  $^{13}\text{C}$ -xylose in their vicinity and switched to another, unlabeled source of carbon.

We also evaluated whether differences in community richness or evenness between tillage managements might contribute to the functional differences seen. No significant differences existed for richness or evenness measures between land managements for either xylose and cellulose incorporator communities at time zero. When extended out to the entire till or no-till community, again no significant differences were seen. However, the richness of communities involved in cellulose degradation was significantly higher than communities involved in xylose degradation.

We also assessed biological replicate microcosms in for both  $^{13}\text{C}$ -cellulose and  $^{13}\text{C}$ -xylose treatments and found that replicates do not have identical incorporators. Till soils generally share more responder OTUs than no-till soils, a result which mimics the unfractionated *in situ* soil, in which no-till samples are more variable as compared to till samples. Spatial heterogeneity may be higher in no-till soils, due to differences in amount organic matter and soil aggregation (Berthrong et al., 2013). The spatial scale at which we sampled soils (across a 10 meter transect) may also exacerbate no-till spatial heterogeneity.

We also acknowledge that fungi are important contributors to cellulose and xylose cycling in soils and may contribute to the functional dissimilarity across tillage

that we see. We have conducted another project that assesses their role in this system.

#### **4.6 Conclusion**

Our study provides evidence towards the functional significance of microbial community composition. Even in the relatively "broad" processes of xylose and cellulose decomposition, we see differences in specific OTUs that incorporate substrate across tillage managements that may be linked to differences in overall C flux in the communities. As microbial parameters are added to carbon models, it is essential to evaluate parameters that may help to explain process variation. Our findings suggest that not only microbial community composition, but also temporal dynamics and spatial heterogeneity of microbial community composition may increase model accuracy and allow for increased understanding of carbon cycling in soils.

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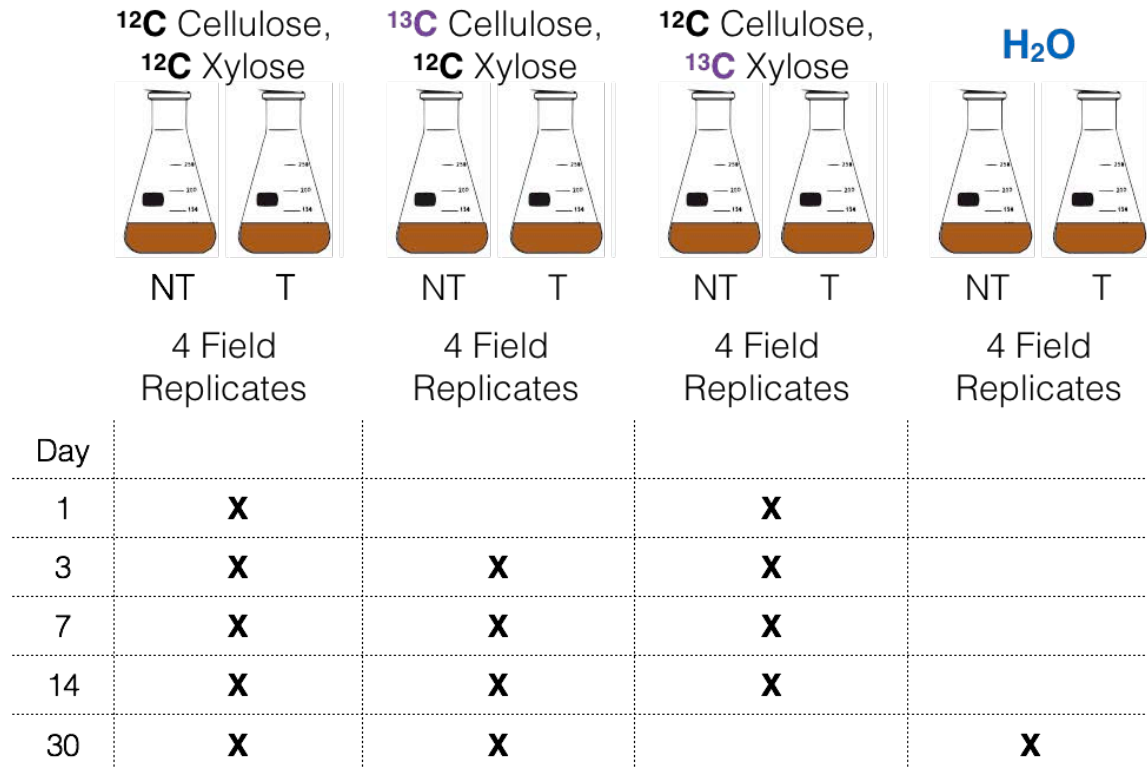
## 4.8 Supplemental Information

### ***Supplemental Note 1 : Mass Balance***

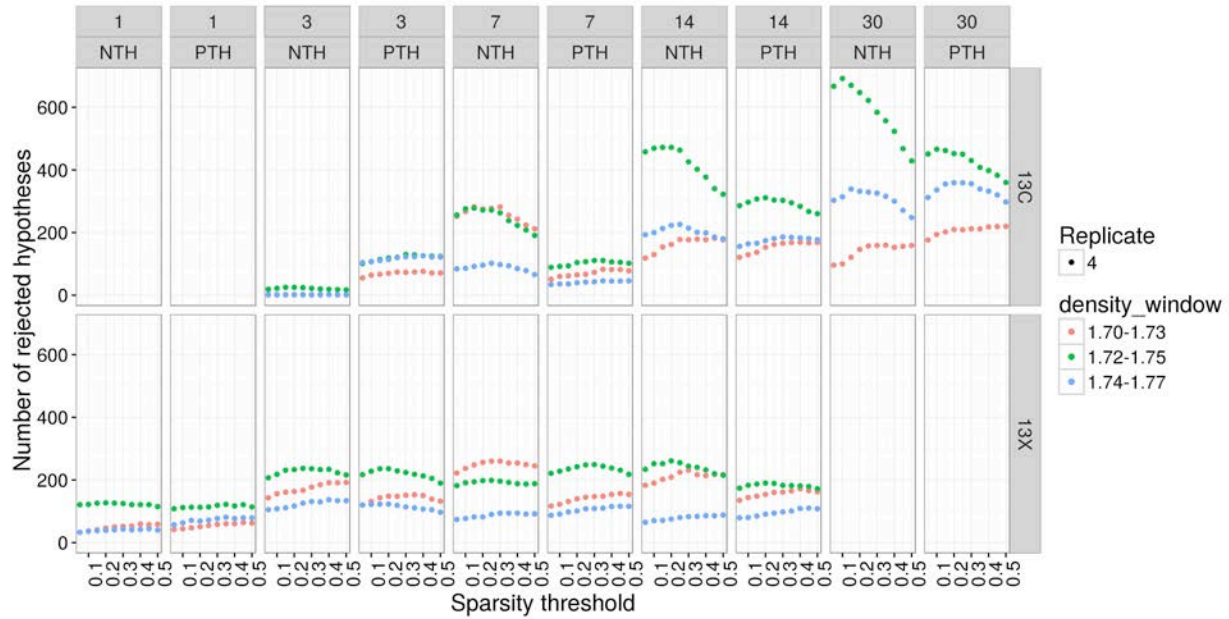
Given the large amount of missing C in the mass balance, especially for the cellulose samples, we hypothesized that some C may have left the soil as methane. To follow this up, we examined our dataset for methanogens and found evidence for their presence. However, we identified too few sequences to be able to run analysis of differential enrichment in  $^{13}\text{C}$  vs  $^{12}\text{C}$  treatments. Design of primers with higher specificity for methanogens may allow us to obtain more information regarding the activity of methanogens in our data and their role in  $^{13}\text{C}$  cellulose decomposition .

Additionally, the  $^{13}\text{C}$  remaining in the soil may not necessarily be cellulose but rather converted into compatible solutes, extracellular polymeric substances, amino acids, or other extensions of microbial biomass. Thus, differences in remaining  $^{13}\text{C}$  in the soil may speak to differences in carbon use efficiency in the no-till vs till soils.

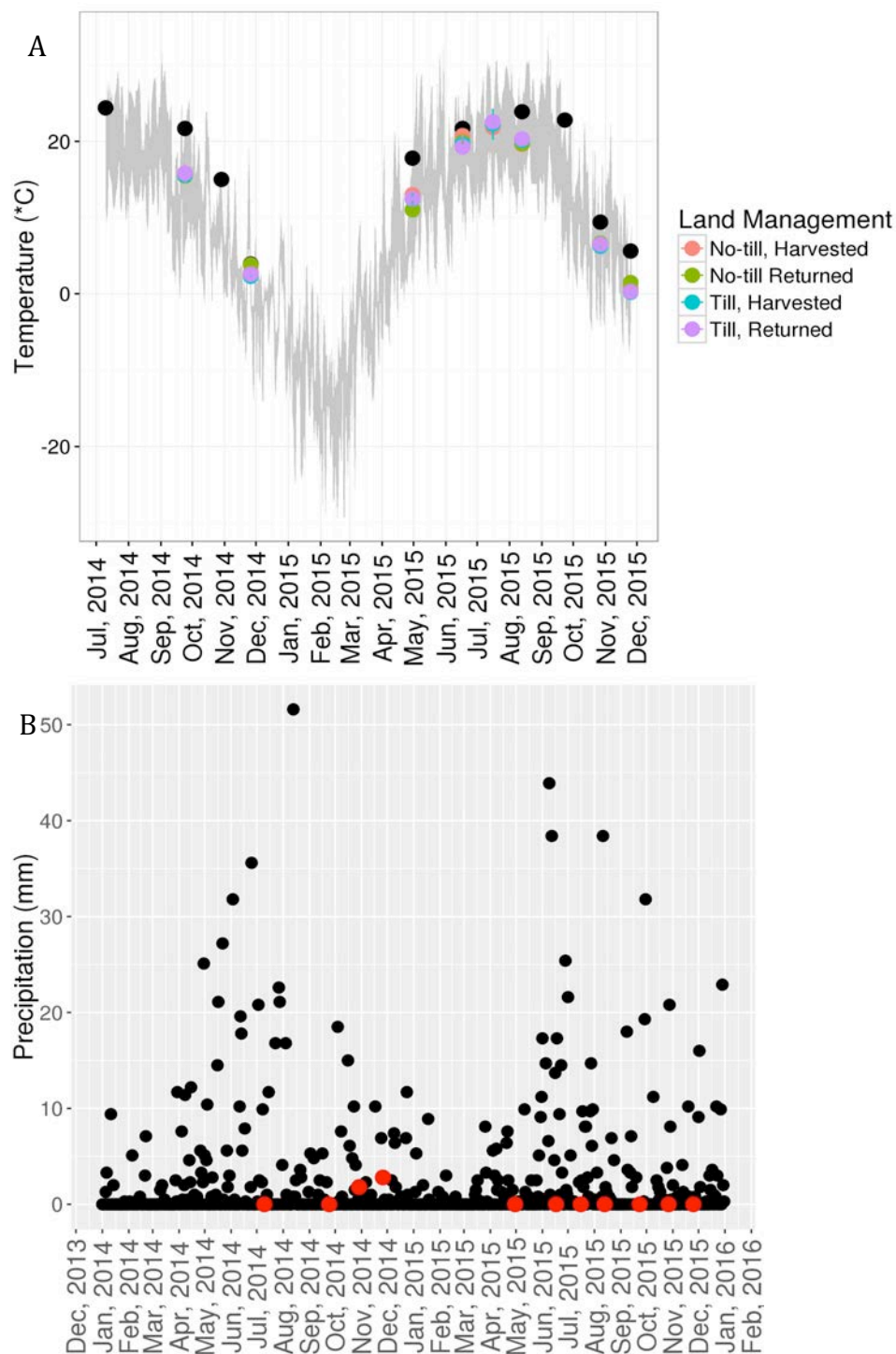
Finally, it is important to keep in mind that the gas data collected represents the entire microbial community within the soil, not just the bacterial community that is the focus of this paper. Therefore, connecting bacterial community dynamics to gas data is not straightforward given the other microbial components that may be contributing or interacting with the bacterial community to result in the observed  $\text{CO}_2$  production patterns.



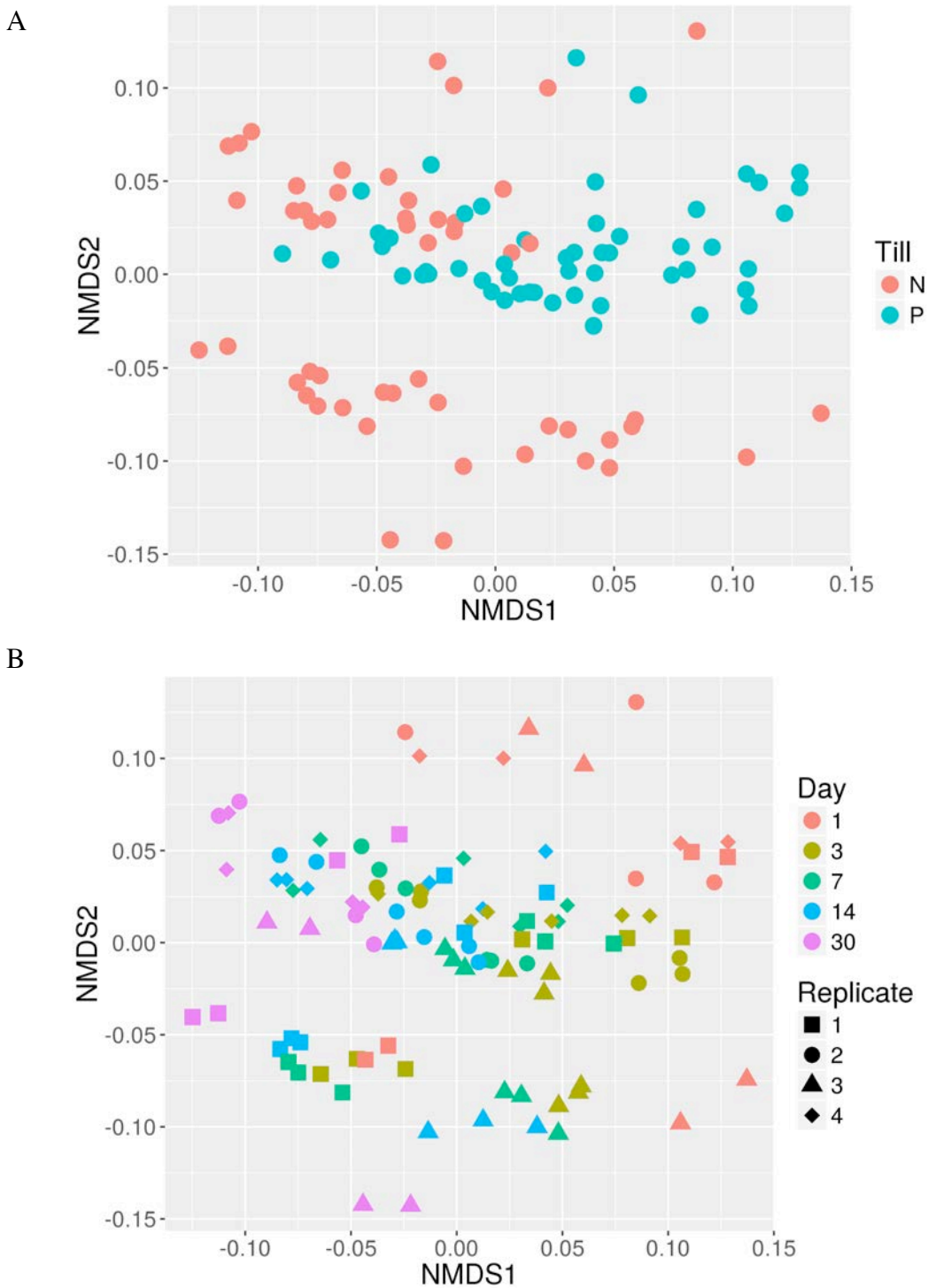
**Supplemental Figure 4.1.** Microcosm set up showing each substrate treatment, tillage management, replicates, and harvesting time point for a total of 112 sample microcosms. NT is an abbreviation for “no-till” and T is an abbreviation for “till”.



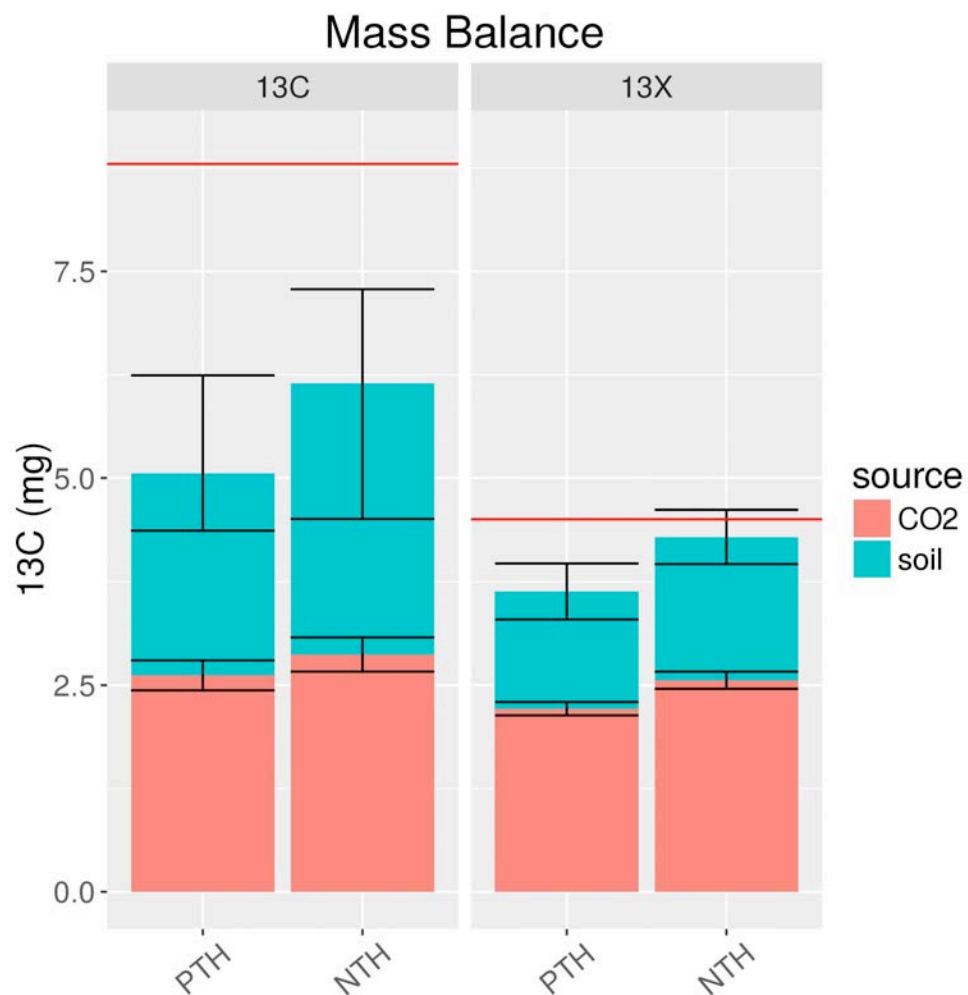
**Supplemental Figure 4.2.** Number of rejected hypothesis ( $p < 0.10$ ) for log<sub>2</sub>-fold change values comparing of OTU abundance in <sup>12</sup>C treatment vs <sup>13</sup>C treatment at each sparsity threshold for each land management, substrate, and time point. The sparsity threshold with the highest number of rejected hypotheses was chosen for each land management, substrate, and time point combination for independent filtering of results



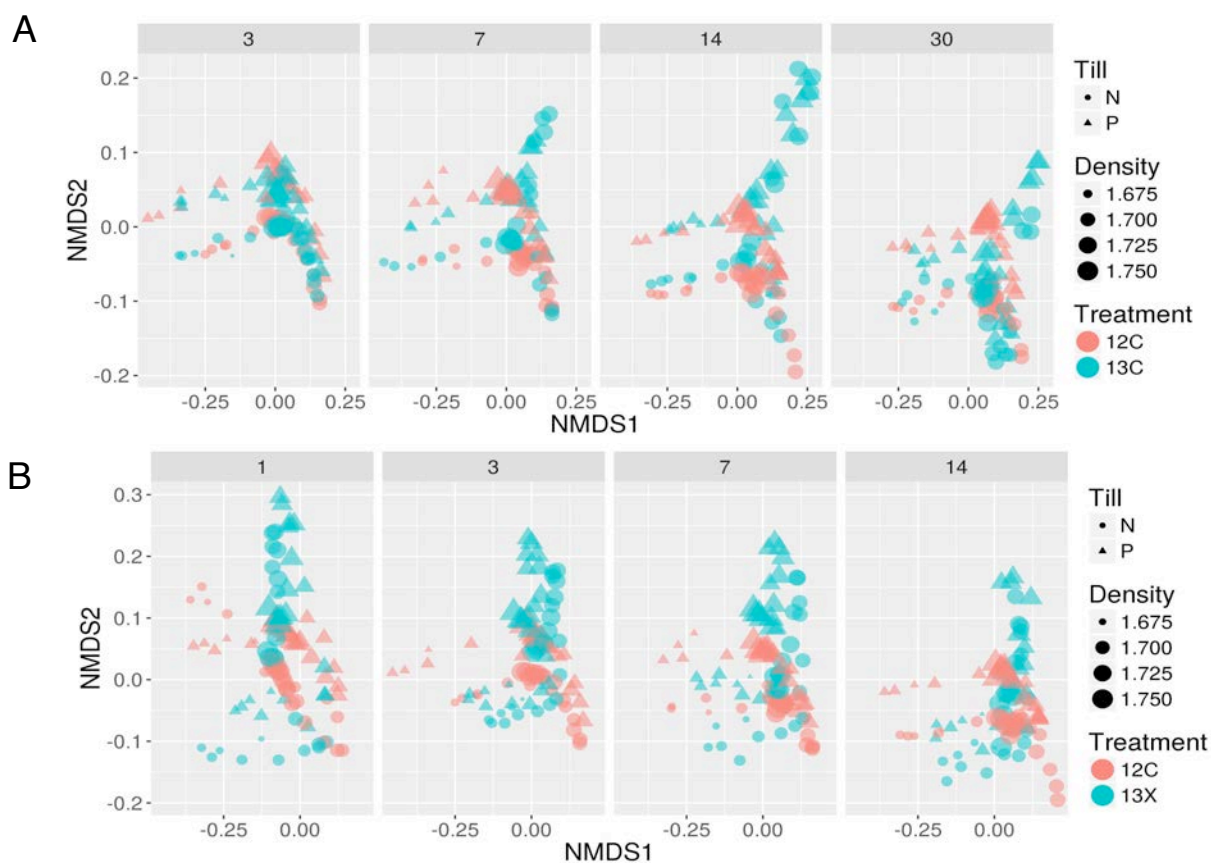
**Supplemental Figure 4.3. (A)** Ambient air temperature range over sampling time period. Black points represent maximum air temperatures on sampling days. Colored points represent soil temperatures for each land management on subset of sampling days (July 2014, October 2014, and September 2015 excluded) **(B)** Precipitation values (mm) for the sampling time period. Red dots indicate precipitation levels on sampling days.



**Supplemental Figure 4.4.** NMDS ordination of weighted UniFrac values for 16S rRNA sequences of individual, unfractionated microcosms of each treatment, soil, and harvesting day combination. Colors represent (A) tillage status or (B) Day of microcosm harvest and shape represents microcosm replicate.



**Supplemental Figure 4.5.** Mass balance of  $^{13}\text{C}$  added for each treatment. X-axis represents different tillage conditions. Figure is faceted by substrate added. Color represents origin of  $^{13}\text{C}$  (red –  $\text{CO}_2$ , blue – soil). Error bars represent 1 standard error and red line represents 100% of  $^{13}\text{C}$  added to each treatment.



**Supplemental Figure 4.6.** NMDS ordination of weighted Unifrac distances for all density fractions of all isotopic treatment – tillage management - day combinations for (A) Cellulose and (B) Xylose. The ordination is faceted by day. Color indicates isotopic treatment, shape indicates land management, and size represents fraction density.

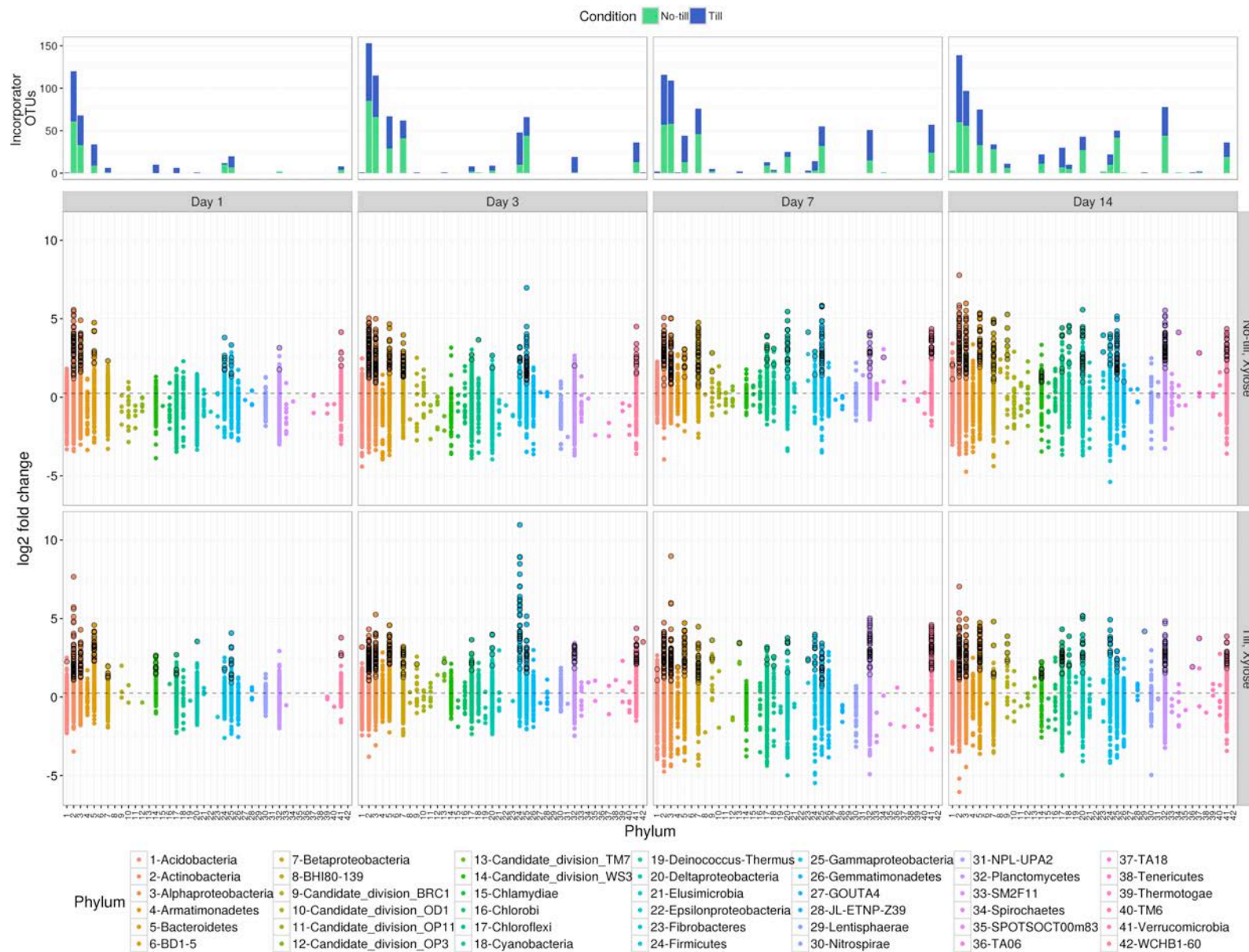
**Supplemental Table 4.1.** PERMANOVA values for comparisons of weighted UniFrac distances for OTUs from corresponding high-density fractions of the  $^{12}\text{C}$  treatment and the  $^{13}\text{C}$  treatment for each soil type, substrate, and time point combination.

Substrate - Tillage	Day	Adjusted P-value	R <sup>2</sup>
Cellulose - Till	3	0.01	0.15
Cellulose - Till	7	0.003	0.21
Cellulose - Till	14	0.001	0.26
Cellulose - Till	30	0.001	0.32
Cellulose - No-till	3	0.40	0.03
Cellulose - No-till	7	0.001	0.28
Cellulose - No-till	14	0.001	0.30
Cellulose - No-till	30	0.001	0.28
Xylose - Till	1	0.001	0.33
Xylose - Till	3	0.001	0.30
Xylose - Till	7	0.001	0.30
Xylose - Till	14	0.001	0.40
Xylose - No-till	1	0.001	0.35
Xylose - No-till	3	0.001	0.41
Xylose - No-till	7	0.001	0.27
Xylose - No-till	14	0.001	0.40

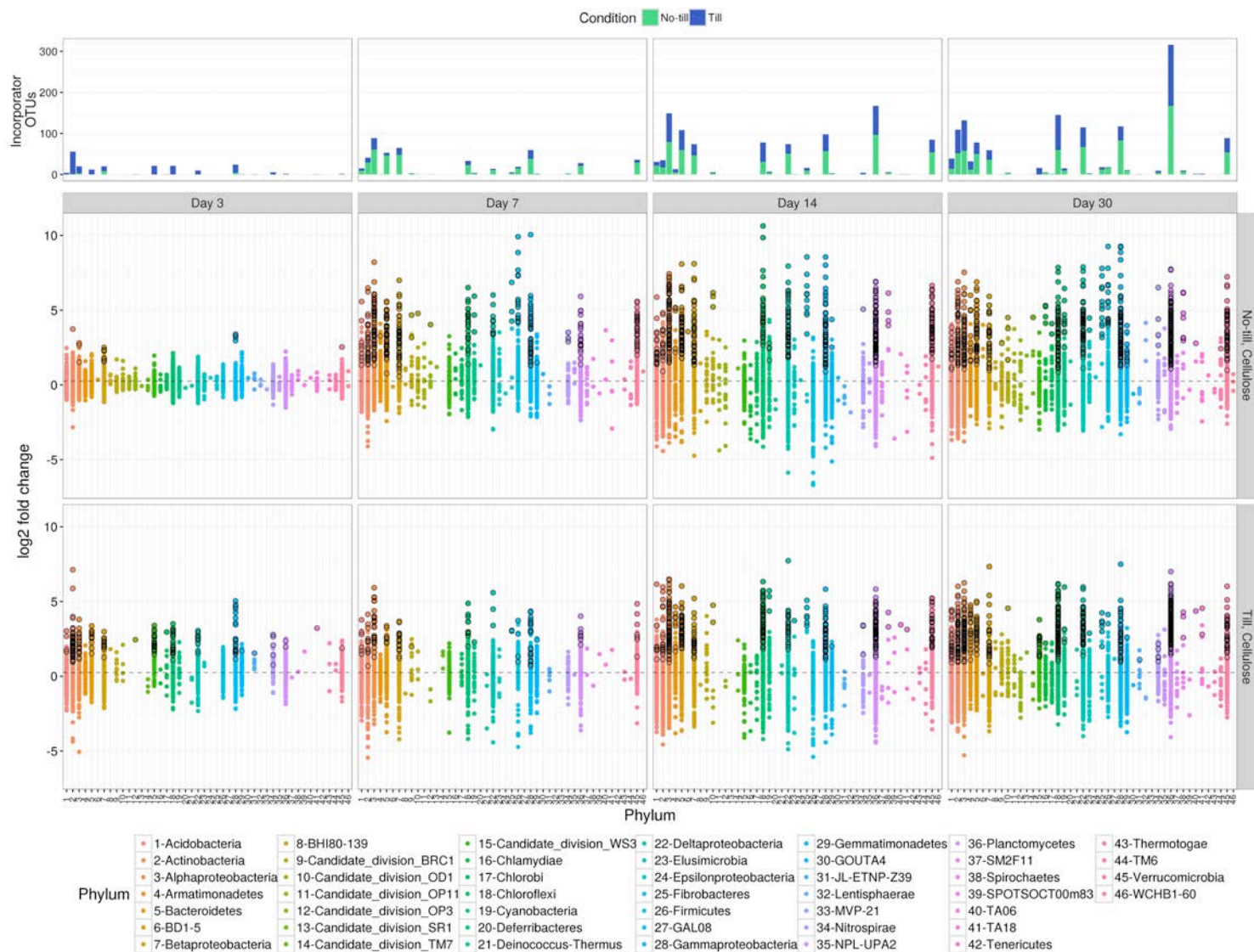


**Supplemental Table 4.2** PERMANOVA values for comparisons of weighted UniFrac distances for OTUs from sets of incorporators in no-till vs. till soils for each substrate and time point combination.

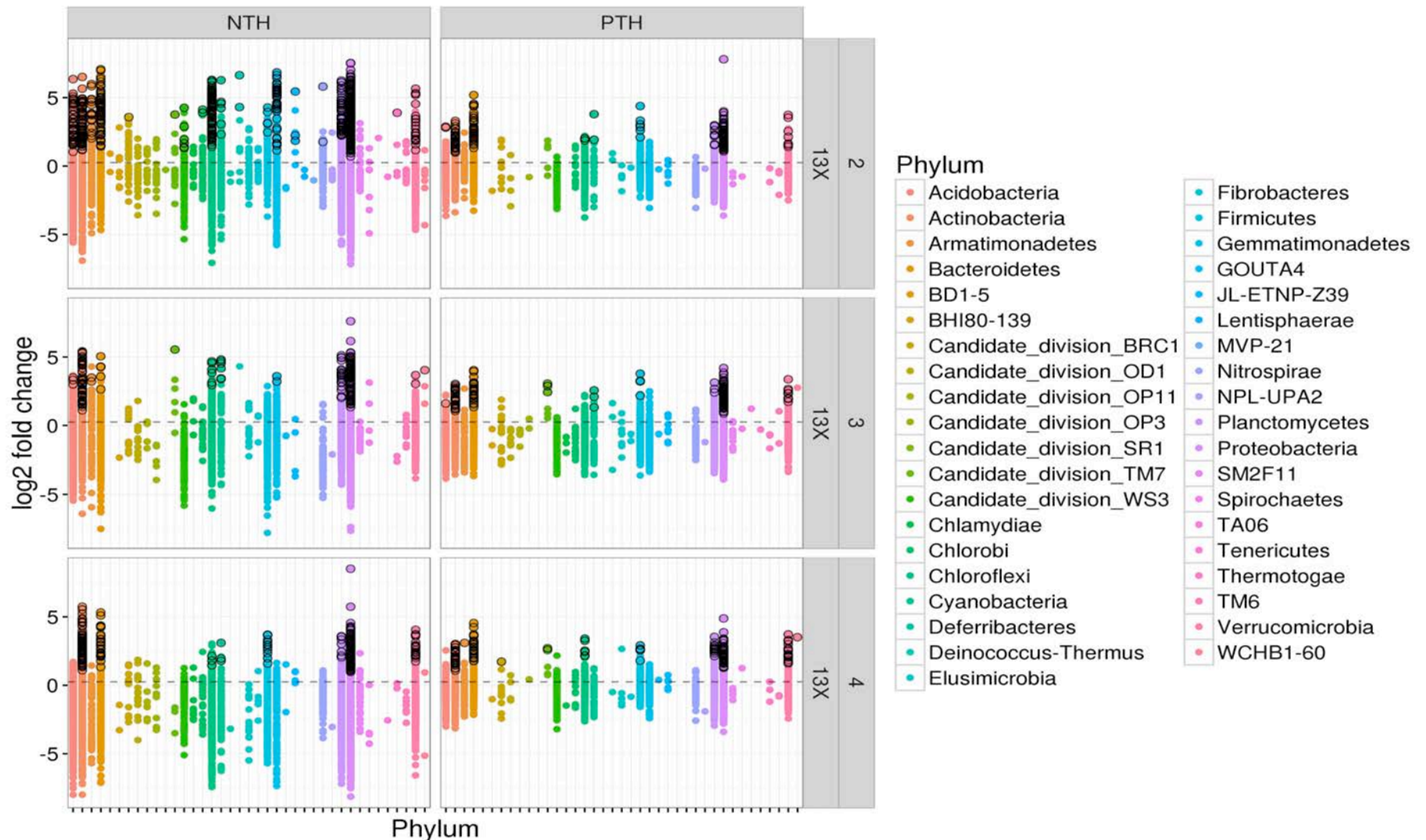
Substrate	Day	Adjusted P-value	R <sup>2</sup>	F-value
Cellulose	3	0.001	0.24	9.4
Cellulose	7	0.02	0.11	3.5
Cellulose	14	0.08	0.07	2.3
Cellulose	30	0.003	0.13	4.6
Xylose	1	0.001	0.35	16.2
Xylose	3	0.001	0.23	8.7
Xylose	7	0.001	0.26	10.3
Xylose	14	0.042	0.08	2.7



**Supplementary Figure 4.7.** Log<sub>2</sub>-fold change values of individual OTUs in <sup>13</sup>C xylose vs. <sup>12</sup>C xylose. Values are organized and colored by phylum and faceted by day and tillage management. OTUs with significant log<sub>2</sub>-fold change values (Wald test,  $p \leq 0.10$ ) are highlighted in black. The top histogram shows number of significant incorporator OTUs (transformed by  $\log_{10} + 1$ ) in each phylum over time, with color representing incorporator status in no-till (green) or till (blue) sample

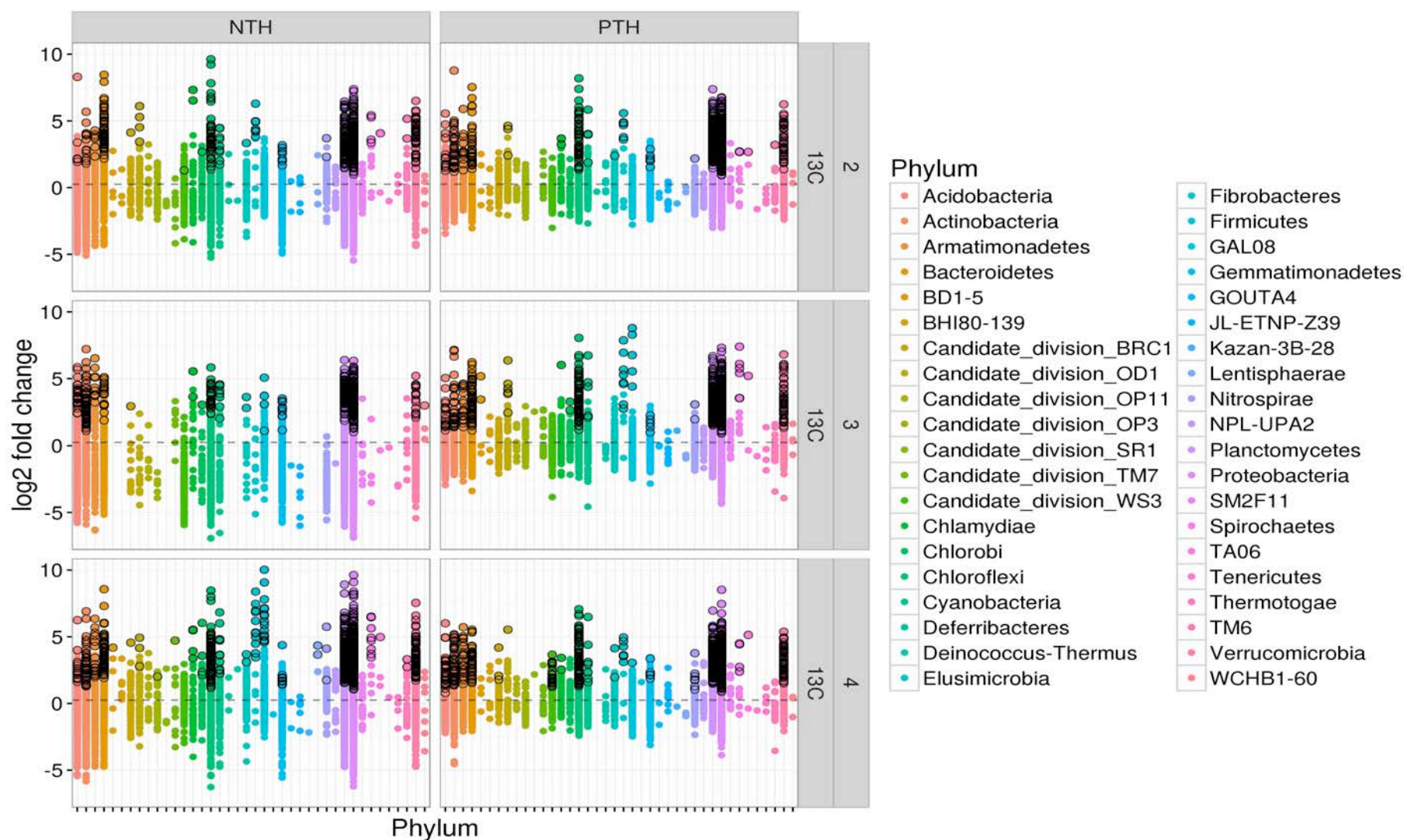


**Supplementary Figure 4.8.** Log<sub>2</sub>-fold change values of individual OTUs in <sup>13</sup>C cellulose vs. <sup>12</sup>C cellulose. Values are organized and colored by phylum and faceted by day and tillage management. OTUs with significant log<sub>2</sub>-fold change values (Wald test,  $p \leq 0.10$ ) are highlighted in black. The top histogram shows number of significant incorporator OTUs (transformed by  $\log_{10} + 1$ ) in each phylum over time, with color representing incorporator status in no-till (green) or till (blue) samples.



**Supplemental Figure 4.9.** Log<sub>2</sub>-fold change values of individual OTUs in <sup>13</sup>C xylose vs. <sup>12</sup>C xylose. Values are organized and colored by phylum and faceted by replicates (2, 3, 4) and tillage management (NTH – no till and PTH - till). OTUs with significant log<sub>2</sub>-fold change values (Wald test, p ≤ 0.10) are highlighted in black.





**Supplemental Figure 4.10.** Log<sub>2</sub>-fold change values of individual OTUs in <sup>13</sup>C cellulose vs. <sup>12</sup>C cellulose. Values are organized and colored by phylum and faceted by replicates (2, 3, 4) and tillage management (NTH – no till and PTH - till). OTUs with significant log<sub>2</sub>-fold change values (Wald test, p <= 0.10) are highlighted in black.

## Conclusion

Humans are landscape engineers who have long manipulated ecosystems for practical end. Oftentimes, though, the long-term effect of land manipulations is not considered. Humans have reconfigured landscapes through agriculture, with tillage and biomass removal as examples of ecosystem manipulation for short-term benefit. In this research, we confirm that tillage and biomass management can impact microbial communities. In the case of fungi, entire phyla are affected, such as the significant increases in *Basidiomycetes* OTU abundances in no-till and biomass returned conditions as compared to tilled and biomass harvested conditions.

We also find that both bacterial and fungal communities are influenced by factors in combination, not just individually. Specifically, we find evidence for seasonal changes in soil carbon inputs affecting microbial communities in different ways across tillage and biomass managements. Soils that are not tilled and that receive returned biomass at the end of the growing season have specific OTUs significantly increasing in abundance, which is not seen in either of the till treatments (biomass return and biomass removed) or the no-till biomass removed treatment. This suggests that interactions between land management and season can have effects on microbial communities.

If these OTUs that grow in abundance in the fall and early are using the returned biomass, as we hypothesize, for energy and carbon content, their activity and growth may impact the cycling of carbon in the soil. In chapter 4, we show evidence for the role of bacterial community composition in the differential cycling of cellulose and xylose in no-till vs tilled soils. Thus, we offer support for the functional significance of soil



microbial communities in carbon cycling. We also show the complexity of interactions of abiotic factors that may result in community shifts seasonally and hypothesize that these seasonal shifts may have functional significance in carbon cycling.

As of now, soil carbon models do not take seasonal impacts on microbial community into account, much less interactions between land management and season. Before such parameters can be placed into models, though, further research is required to adequately characterize impacts of these parameters and their interactions on microbial communities in various soils types and climates. Additionally, more evidence is needed to confirm the relationship between microbial community structure and function (in carbon cycling) in relation to these variables. Tools such as high-throughput sequencing and high-resolution stable isotope probing (HR-SIP), when combined with effective experimental set-ups, can produce such evidence.

At this point in time, microbial ecology studies relying on sequencing methods are limited from analysis of compositional data. This study is no exception. We are able to track relative abundance changes for specific OTUs, but we do not know whether these changes are a result of increased or decreased abundance of that OTU or of a decrease or increase in abundance of other OTUs. The use of ratio-based methods, such as the DESeq2 analysis used in this study allows for more stringent and more comparative analysis of relative abundances. However, future studies would benefit from quantitative measures of microbial DNA in samples that would allow the calculation of actual abundance of microbial OTUs. One such method involves adding an internal standard to soils to more accurately quantify DNA obtained through the DNA extraction

process that then proceeds to sequencing (Smets et al., 2016). Additionally, this study would benefit from measurement of incorporation of  $^{13}\text{C}$  into microbial biomass, allowing us to calculate carbon use efficiency for the set of OTUs involved in cellulose or xylose decomposition.

The study of carbon cycling in soils also lacks spatial resolution. Using HR-SIP, we can identify OTUs with the functional potential to incorporate C from cellulose or xylose in our microcosms. It is thought that some of these OTUs may be working together to break down these substrates, especially cellulose (Wilson, 2011). However, our data does not allow us to see the spatial organization of the incorporators we identify. Perhaps a set of incorporators occupies the same microaggregate and is degrading cellulose through formation of a biofilm community. Another OTU may be isolated from others, but has the all the cellular machinery required for cellulose or xylose degradation, and grows and multiplies in isolation. Our lack of spatial knowledge makes it difficult to produce convincing evidence for interaction between OTUs involved in carbon cycling.

Continued research at long-term field sites testing tillage and biomass management is essential for studies looking at the influences of interacting factors on microbial community structure and the functional impacts of shifts in structure. Combining HR-SIP with measurements of carbon use efficiency, quantitative DNA analysis, and data regarding spatial relations between soil microbes is one of the next steps in more holistically understanding carbon cycling in soils. Technology is advancing that will hopefully make such a study possible in the near future.

However, much additional work is still possible with the datasets collected in this thesis study. We have results for the HR-SIP experiment conducted with the fungal community that are in the analysis process. Preliminary results from the study indicate a similar difference in beta diversity of incorporator OTUs between till and not-till soils, especially for the cellulose substrate. Thus, fungal community differences may also contribute to functional differences in carbon cycling. Another future goal is to track OTUs identified as incorporators of  $^{13}\text{C}$ -cellulose and  $^{13}\text{C}$ -xylose across the 2-year seasonal dataset. Are OTUs that have the capability of incorporating carbon from cellulose or xylose consistent in abundance throughout the year or do they increase in abundance at certain points of the year? Are they present in all agricultural treatments or only in certain treatments? If we are able to group incorporators into categories based their seasonal and treatment abundance patterns, we may be able to learn more about microbial niches in the soil environment and relate them back to functional potential in carbon cycling.

I use the phrase “functional potential” because the HR-SIP experiment was conducted in lab-based microcosms, under conditions that are not identical to conditions in the soil environment. Soils in the experiment were sieved and did not experience temperature or moisture changes as they would have in the field. However, we tried to keep conditions similar to the soil environment. We chose to add carbon as a corn biomass simulant, with several sources of carbon available to the microbial community as would occur in the soil environment. The concentration of carbon added was low and environmentally-relevant. Therefore, our results can be taken as an indication of the

functional potential of microbial communities.

This study provides evidence for the importance in understanding not only the effect of land management and time on microbial communities, but also understanding that impacts of land management can change over time. We also find that the impacts of tillage on bacterial community structure can result in functional changes in carbon cycling within the soil. Thus, this study adds to growing evidence of the functional importance of bacterial community structure.

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